1997

Structure-function relationships in porcine fructose-1, 6-bisphosphatase

Rulin Zhang
Iowa State University

INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Structure-function relationships in porcine fructose-1,6-bisphosphatase

by

Rulin Zhang

A dissertation submitted to the graduate facuty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Biochemistry
Major Professor: Herbert J. Fromm

Iowa State University
Ames, Iowa
1997

Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Rulin Zhang

has met the dissertation requirements of

Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program

Signature was redacted for privacy.

For the Graduate College

DEDICATION

To my parents, who gave me life and nurtured me;

To my teachers, who gave me knowledge and encouragements;

To my wife and my daughter, for their sacrifice and patience

TABLE OF CONTENTS

ABSTRACT

Fructose-1,6-bisphosphatase (FBPase) plays a crucial role in the regulation of gluconeogenesis. It is a homotetramer with a subunit molecular mass of 37 kDa. Each subunit has one substrate and three metal binding sites. Site-directed mutagenesis were carried out to replace amino acid residues which are thought to play important roles in structure integrity and functioning of the enzyme. Particular emphasis was placed on residues in the substrate (N212,R243, Y244, Y264, K274) and metal (R276, E280) binding sites as well as the residues at the C1C2 (C3C4) interface (S123, D127, C128, Y258 and R243 from adjacent chain). The mutant and wild-type FBPases were purified to homogeneity and characterized by initial-rate kinetics and circular dichroism spectrometry (CD). There were no discernible differences between the secondary structures of the wild-type and the mutant enzymes on the basis of CD data. Altering G122 to alanine caused a significant decrease in the enzyme's activity and affinity for Mg^{2+}. Most importantly, the mutation caused the total loss of cooperativity for Mg^{2+}. Thus, it is believed that G122 is critical for Mg^{2+} cooperativity and binding as well as for enzyme activity. Together with other residues, R276 defines metal site 3 and E280 defines both metal site 1 and 3. Replacement of R276 with methionine caused the total loss of Mg^{2+} cooperativity and changed the kinetic mechanism from rapid equilibrium random (or steady-state ordered) to rapid

equilibrium ordered, in which the substrate adds to the enzyme before Mg^{2+} and all steps equilibrate rapidly relative to the conversion of the ternary complex. In addition, the mutation abolished the inhibition effects of monovalent cations with the enzyme. When E280 was mutated to glutamine, the enzyme's activity was decreased over 1000-fold, and the mutation changed K^+ to a noncompetitive inhibitor with respect to Mg^{2+}. K274, Y264, R243, Y244, and N212 are involved in substrate binding; S123, D127, C128, and residues Y258 and R243 from adjacent chain are located at the C1C2 (C3C4) interface. The mutant FBPases, K274L, Y264F, Y244F, Y258F, R243M, S123A, D127T, and C128G, changed ligand binding affinities.

In addition, a kinetic model was developed to account the kinetics and mechanisms of activation and inhibition of FBPase by monovalent cations.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

It is well known that fructose-1,6-bisphosphatase (FBPase) is a crucial step in the regulation of gluconeogenesis (Underwood & Newsholme, 1965; McGilvay, 1964; Krebs, 1963; Pilkis et al., 1988). The enzyme catalyzes the hydrolysis of fructose 1,6-bisphosphate (FBP) to fructose 6-phosphate (F6P) and inorganic phosphate and exhibits a rapid equilibrium random Bi Bi kinetic mechanism (Liu & Fromm, 1990). The reaction is competitively inhibited by fructose 2,6-bisphosphate (F26P) and noncompetitively inhibited by AMP (Liu & Fromm, 1990). These two compounds are also involved in the activation of phosphofructokinase (Hers & Van Schaftingen, 1982, Pilkis et al., 1988). Therefore, it is thought that F26P plays crucial roles in the regulation of glycolysis and gluconeogenesis. Although the concentration of AMP remains relatively constant in the cell due to the activity of adenylate kinase, AMP is also an important element in the regulation of FBPase because it acts synergistically with F26P to inhibit FBPase (Hers & Van Schaftingen, 1982). The role of AMP is thought to prevent divalent metal binding to FBPase, and divalent cations such as Mg^{2+} and Mn^{2+} are absolutely required for FBPase activity (Liu & Fromm, 1990; Scheffer & Fromm, 1986). One of the functions of F26P is to keep AMP on the enzyme (make it stickier) (Liu & Fromm, 1988), thus,

enhancing the action of AMP.

FBPase has long been recognized to be a metal-requiring enzyme (Gomori, 1943). Univalent cations can activate the enzyme (Hubert et al., 1970), but divalent cations are absolutely required for enzyme activity (Gomori, 1943). The crystallographic structure, reported by the Lipscomb group (Zhang et al., 1993), suggests that two divalent cations are bound at the metal-binding site. Benkovic et al. (1978,1982) suggested that catalysis requires the sequential addition of metal and substrate in the order, structural metal, substrate, and catalytic metal to form a catalytically competent quaternary complex of enzyme-M_1-M_2-FBP. Nimmo and Tipton (1975a, 1975b) showed that pH could affect the divalent metal kinetics of bovine liver FBPase; i.e., the plots of velocity versus Mg^{2+} are sigmoidal at neutral pH, but they are hyperbolic at pH 9.6. Furthermore, cooperativity with respect to metal ion binding and its activation of the mammalian FBPase reaction has long been recognized (Benkovic & deMaine, 1982; Benkovic et al., 1978). The cooperativity is enhanced by K^+ (Hubert et al., 1970) Recently, Chen et al. (1993) and El-Maghrabi et al. (1993) carried out site-directed mutagenesis experiments in the metal binding sites of porcine FBPase. Their investigations support the suggestion that there are two metal binding sites associated with FBPase. Zhang et al.(1995) showed that porcine liver FPBase lost Mg^{2+} cooperativity at pH 7.5 when Gly 122 was mutated to alanine.

FBPase from mammalian liver and kidney, two highly gluconeogenic tissues, is a homotetramer (Marcus et al., 1982). Alteration of the amino acid residues in the metal binding site can affect, not only the cooperativity and ligand affinity of metal ions, but also affects the enzyme's affinity for AMP (Chen et al., 1993). These observations suggest that metal binding sites and AMP binding sites may somehow communicate with each other.

A monovalent cation is needed for the enzyme to achieve its maximal activity (Hubert et al., 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Marcus & Hosey, 1980). Among the monovalent cations studied, K^+, NH_4^+, and Tl^+ are the best activators, whereas Li^+ is a strong inhibitor; however, how these monovalent cations affect the activity of FBPase remains unclear. Tejweni et al. (1976) proposed that the activation effect of K^+ was to overcome inhibition of the enzyme by high concentrations of divalent metal ions. Xu et al. (1993) suggested that activation by monovalent cations might result from the binding of K^+ at a site distinct from the catalytic site. Marcus (1975) reported that activation of FBPase by K^+ could be abolished by modifying arginyl residues with 2,3-butanedione in the presence of AMP. He also found that only one arginyl residue per subunit played an essential role in monovalent cation activation of the enzyme. This arginyl residue was thought to be in the substrate binding site on the basis of the fact that no loss of monovalent

cation activation occurred when modification was carried out in the presence of AMP plus the substrate. Very probably, this residue is Arg 276, which forms a salt-bridge in the absence of divalent cations (Zhang et al., 1993). This arginyl residue plays important roles in both enzyme activity and Mg^{2+} cooperativity, and in determining the kinetic mechanism of FBPase (Zhang & Fromm, 1995).

Phosphoryl transfer enzymes form one of the largest families of biological catalysts (Knowles, 1980). Most of these enzymes, if not all, are activated and/or inhibited by monovalent cations, yet the mechanism of the action of monovalent cations is not fully understood. FBPase, which plays an important role in the regulation of gluconeogenesis (Krebs, 1963; Marcus, 1981; Hers & Hue, 1983; Pilkis et al., 1988), belongs to this family.

Since the finding of the activation effect of K^+ on pyruvate kinase (Boyer et al., 1942), monovalent cations have been shown to regulate many enzymes (Suelter, 1970). Some of these enzymes require a monovalent cation for activity, others do not. The roles of monovalent cations in enzyme catalysis and/or regulation are not clear. FBPase, like all other phosphotransferases, can be affected by monovalent cations (Marcus, 1975; Hubert et al., 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Nakashima & Tuboi, 1976; Marcus & Hosey, 1980; Zhang et al., 1996). Among the monovalent cations studied, K^+, NH_4^+, and Tl^+ are the best activators,

whereas Li^+ is a strong inhibitor; however, how these monovalent cations affect the activity of FBPase is not fully understood. The activation effect of K^+ was thought to be the result of reversing the inhibition caused by high concentrations of divalent metal ions (Tejwani et al., 1976) or from the binding of K^+ at an allosteric site (Xu et al., 1993). Marcus (1975) reported that activation of FBPase by K^+ could be abolished by modifying an arginyl residue at the active site. These findings indicated that K^+ ion probably binds at the active site. Hubert et al. (1970) reported that K^+ ions could affect the sigmoidicity of the saturation curves of divalent cations.

Recently, x-ray diffraction studies (Villeret et al., 1995) demonstrated that K^+ and Tl^+ ions bind at three metal sites located at the active site of FBPase. Two sites, defined as sites 1 and 2, correspond to the divalent metal sites previously defined (Zhang et al., 1993). The third site is thought to be specific for K^+ and Tl^+, is 3.4 Å away from site 1, and is defined by Glu 280, Arg 276, and the 1-phosphoryl group of the substrate (Villeret et al., 1995). Therefore, Glu 280 and Arg 276 are thought to be critical to the kinetics of monovalent cation action with FBPase. It is known that Glu 280 and Arg 276 are important for the activity of FBPase (Chen et al., 1993; Zhang & Fromm, 1995). Furthermore, Arg 276 determines Mg^{2+} cooperativity and the kinetic mechanism (Zhang & Fromm, 1995), and Glu 280 is

essential for K^+ binding at site 3 (Zhang et al., 1996). These results support the notion that one of the roles of monovalent cations is to "bridge substrate and the important residues" of enzymes.

FBPase is a homotetramer with D_2 symmetry. X-ray diffraction studies showed that this enzyme is a dimmer of dimmers and exists in two quaternary conformations, the active R form and the inactive T form (ref.). The binding of AMP may lock the enzyme to T state (ref.). It is observed that helix H4 (residues 123-127) is subject to conformational changes during catalysis. Also, H4 is better defined in the quaternary complex of Enzyme-F26P-AMP-Zn^{2+} than in the R-forms (11). In the quaternary structure, K274, S123, S124, and R243 (from adjacent chain) bind to F26P much tighter (Fig.1B) compare with that in Enzyme-F26P complex (Fig.1A). Most interestingly, Y258 from the adjacent chain turns greater than 90^{o} around C_{α}-C_{β} bond in the quaternary complex and its hydroxyl group form hydrogen bonds to the backbone carbonyl of S124 and amide of C128 (see Fig.1B). This was not observed in other T-forms. Therefore, this residue and residues of H4 are thought to be involved in the synergism between F26P and AMP.

Dissertation organization

This dissertation contains eight chapters. Chapter 1 describes the research problems and previous work. Chapters 2 to 5 are papers published in peer reviewed journals. Chapters

6 and 7 are manuscripts submitted to scholarly journals for publication. Chapter 2 discusses the loss of Mg^{2+} cooperativity with FBPase by replacing G122 with alanine. Chapter 3 describes the changes of Mg^{2+} cooperativity and kinetic mechanism with the enzyme by mutating R276 to methionine. Chapter 4 is about the kinetics and mechanisms of activation and inhibition of the enzyme by monovalent cations. Chapter 5 describes the mutational effect on the substrate binding site. Chapter 6 describes the modeling and mutational effects on metal site 3. Chapter 7 discusses the effects of mutations at C1C2 (C3C4) interface in the active site domain of FBPase on ligand binding and the synergism between AMP and F26P. Chapter 8 contains a general conclusion which summarizes the research results. In Chapter 8, future research direction is also discussed.

References

Benkovic,P.A., Caperelli,C.A., deMaine,M.M., and Benkovic, S.J. (1978) Proc. Natl. Acad. Sci. U.S.A., **75**, 2185-2189.

Benkovic, S. J., & deMaine, M. M. (1982) Adv. Enzymol. Relat. Areas Mol. Biol. **53**, 45-82.

Boyer, P. D., Lardy, H. A., & Phillips, P. H. (1942) J. Biol. Chem. **146**, 673-682.

Chen, L., Remesh, H., Chen, M., & Fromm, H. J. (1993) Arch. Biochem. Biophys. **307**, 350-354.

Colombo,G., & Marcus,F.(1973) J. Biol. Chem. **248**, 2743-2745.

El-Maghrabi, M.R., Gidh-Jain,M., Austin,L.R., and Pilkis, S.J. (1993) J. Biol. chem., **268**, 9466-9472.

Gomori, G. (1943) J. Biol. Chem. **148**, 139-149.

Hers, H. G., & Hue, L. (1983) Annu. Rev. Biochem. **52**, 617-653.

Hers, H. G., & Van Schaftingen, E. (1982) Biochem. J. **206**, 1-12.

Hubert, E., Villanueva, J., Gonzalez, A. M., & Marcus, F. (1970) Arch. Biochem. Biophys. **138**, 590-597.

Knowles, J. R. (1980) Annu. Rev. Biochem. **49**, 877-919.

Krebs, H. A. (1963) in Advances in Enzyme Regulation (Weber, G., ed) Vol. 1, pp. 385-400, Pergamon Press Ltd., London.

Liu, F., & Fromm, H. J. (1990) J. Biol. Chem. **265**, 7401-7406.

Liu,F., and Fromm,H.J.(1988)J. Biol. Chem. **263**,9122-9128.

Marcus, F. (1975) Biochemistry **14**, 3916-3921.

Marcus, F. (1981) in The Regulation of Carbohydrate Formation and Utilization in Mammals (Veneziabe, C.M., ed.) pp.269-290, University Park Press, Baltimore.

Marcus, F., Edelstein, L., Reardon, I., & Heinrikson, R.L. (1982) Proc. Natl. Acad. Sci. U. S. A. **79**, 7161-7165.

Marcus, F., & Hosey, M. M. (1980) J. Biol. Chem. **255**, 2481-2486.

McGilvay,R.W. (1964) Fructose-1,6-diphosphatase and its role in Gluconeogenesis (McGilvay, R.W., and Pogell, B.M.,Eds). Port City Press Inc, Baltimore.Nakashima, K., & Tuboi, S. (1976) J. Biol. Chem. **251**, 4315-4321.

Nimmo, H. G., & Tipton, K. F. (1975a) Eur. J. Biochem. **58**, 567-574.

Nimmo, H. G., & Tipton, K. F. (1975b) Eur. J. Biochem. **58**, 575-585.

Pilkis, S. J., El-Maghrabi, M. R., & Claus, T. H. (1988) Annu. Rev. Biochem. **57**, 755-783.

Scheffler,J.E., and Fromm,H.J.(1986)Biochemistry **25**, 6659-6665.

Suelter, C. H. (1970) Science **168**, 789-795.

Tejwani, G. (1983) Adv. Enzymol. Relat. Areas Mol. Bio. **54**, 121-193.

Tejwani, G. A., Pedrosa, F. O., Pontremoli, S., & Horecker, B. L. (1976) Arch. Biochem. Biophys. **177**, 255-264.

Underwood,A.H., and Newsholme,E.A. (1965) Biochem. J. **95**, 767-774.Villeret, V., Huang, S., Fromm, H. J., & Lipscomb, W. N. (1995) Proc. Natl. Acad. Sci. U. S. A. **92**, 8916-8920 .

Villanueva, J., & Marcus, F. (1974) J. Biol. Chem. **249**, 745-749.

Xu, G., Yu, Z., Hu, G., & Cao, H. (1993) Biochem. Biophys. Res. Commun. **194**, 1483-1490.

Zhang, R., & Fromm, H. J. (1995) Biochemistry **34**, 8190-8195.

Zhang, Y., Liang, J.-Y., Huang, S., Ke, H., & Lipscomb, W. N. (1993) Biochemistry **32**, 1844-1857.

Zhang, R., Villeret, V., Lipscomb, W. N., & Fromm, H. J. (1996) Biochemistry 35, 3038-3043.

CHAPTER 2. GLYCINE 122 IS ESSENTIAL FOR COOPERATIVITY AND BINDING OF MG^{2+} TO PORCINE FRUCTOSE-1,6-BISPHOSPHATASE[1]

A paper published in Journal of Biological Chemistry[2]

Rulin Zhang[3,4], Lirong Chen[3], Vincent Villeret[5]
and Herbert J. Fromm[3,6]

Abstract

Site-directed mutagenesis of an amino acid residue in the substrate binding site of porcine fructose-1,6-bisphosphatase was carried out based on the crystal structure of the enzyme [Zhang, Y., Liang, J.-Y., Huang, S., Ke, H., and Lipscomb, W.N. (1993) Biochemistry, 32, 1844-1857]. A mutant enzyme form of fructose-1,6-bisphosphatase, G122A, was purified and characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis, circular dichroism spectrometry (CD), and initial-rate kinetics. There were no discernible differences between the secondary structures of the wild-type and the

---

[1]This research was supported in part by Research Grants NS 10546 and GM06920 from the National Institute of Health, United State Public Health Service, and Grant MCB-9218763 from the National Science Foundation. This is Journal Paper J-15979 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Project 2575.
[2]Reprinted with permission of J.Biol.Chem., 1995, **270**, 54-58.
[3]Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011.
[4]Primary researcher and author under the supervision of Herbert J. Fromm.
[5]Gibbs Chemical Laboratory, Harvard University, Cambridge, Massachusetts 02138.
[6]To whom all correspondence should be addressed.

mutant enzyme on the basis of CD data. Altering Gly122 to alanine caused a significant decrease in the enzyme's activity and affinity for Mg^{2+}. The K_{cat} for this mutant enzyme was only about 5% of that of wild-type fructose-1,6-bisphosphatase, and the Ka for Mg^{2+} was about 15-fold higher than that of the wild-type enzyme. The K_i for AMP was increased 77-fold in the case of the mutant enzyme; however, the Hill coefficient was unaltered. Most importantly, it was observed that replacement of Gly122 with alanine caused the total loss of cooperativity for Mg^{2+}. It is concluded that Gly122 is essential for Mg^{2+} cooperativity and important for binding of Mg^{2+} and AMP as well as for enzyme activity.

Introduction

It is well known that fructose-1,6-bisphosphatase (FBPase) is a crucial step in the regulation of gluconeogenesis (1-3). The enzyme catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P_2) to fructose 6-phosphate (Fru-6-P) and inorganic phosphate and exhibits a rapid equilibrium random Bi Bi kinetic mechanism (4). The reaction is competitively inhibited by fructose 2,6-bisphosphate (Fru-2,6-P_2) and noncompetitively inhibited by AMP(4). These two compounds are also involved in the activation of phosphofructokinase (5). Therefore, it is thought that Fru-2,6-P_2 plays crucial roles in the regulation of glycolysis and gluconeogenesis. AMP is also an important element in the

regulation of FBPase because it acts synergistically with Fru-2,6-P_2 to inhibit FBPase (5). The role of AMP is thought to prevent divalent metal binding to FBPase, and divalent cations such as Mg^{2+} and Mn^{2+} are absolutely required for FBPase activity (4,6). One of the functions of Fru-2,6-P_2 is to keep AMP on the enzyme (make it stickier) (7), thus, enhancing the action of AMP.

FBPase has long been recognized to be a metal-requiring enzyme (8). Univalent cations can activate the enzyme (9), but divalent cations are absolutely required for enzyme activity (8). The crystallographic structure, reported by the Lipscomb group (10), suggests that two divalent cations are bound at the metal-binding site. Benkovic et al. (11,12) suggested that catalysis requires the sequential addition of metal and substrate in the order, structural metal, substrate, and catalytic metal to form a catalytically competent quaternary complex of enzyme-M_1-M_2-Fru-1,6-P_2. Nimmo and Tipton (13,14) showed that pH could affect the divalent metal kinetics of bovine liver FBPase; i.e., the plots of velocity versus Mg^{2+} are sigmoidal at neutral pH, but they are hyperbolic at pH 9.6. Furthermore, cooperativity with respect to metal ion binding and its activation of the mammalian FBPase reaction has long been recognized (11, 13-15). Recently, Chen et al. (16) and El-Maghrabi et al. (17) carried out site-directed mutagenesis experiments in the metal binding sites of porcine FBPase. Their investigations support

the suggestion that there are two metal binding sites associated with FBPase.

FBPase from mammalian liver and kidney, two highly gluconeogenic tissues, is a homotetramer (18). Alteration of the amino acid residues in the metal binding site can affect, not only the cooperativity and ligand affinity of metal ions, but also affects the enzyme's affinity for AMP (16). These observations suggest that metal binding sites and AMP binding sites may somehow communicate with each other.

X-ray diffraction results of FBPase have pinpointed a number of amino acid residues that are associated with substrate binding (10). These findings (10) suggest that the main chain NH group of the Gly122 residue forms a bifurcated hydrogen bond to the ester oxygen and a 1-phosphoryl oxygen of the substrate. Thus, this residue is postulated to have the function of fixing the 1-phosphoryl group in the required position for metal binding and catalysis.

To gain some insight into the residues involved in substrate binding and catalysis, we have prepared a mutant of FBPase at position 122 by site-directed mutagenesis and have studied its properties. In this study, we report that replacement of Gly122 with alanine causes the total loss of cooperativity of Mg^{2+} and a very significant decreases in enzyme activity, AMP binding and affinity for metal ions.

Experimental Procedures

Materials--NADP, fructose 1,6-bisphosphate (Fru-1,6-P_2), fructose 2,6-bisphosphate (Fru-2,6-P_2), AMP, Hepes, and Tris were purchased from Sigma (St. Louis, MO). Glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim (Indianapolis, IN). Distilled deionized water was used in all experiments. All other reagents were of the highest purity available commercially. Recombinant and mutant forms of porcine liver FBPase were prepared and purified as described elsewhere (19) with slight modifications. Mutant forms of the enzyme were obtained in yields comparable to the wild-type enzyme. Porcine liver and kidney FBPase are identical in their primary sequences (19). There was no measurable endogenous expression of wild-type FBPase in the absence of added isopropyl-ß-D-thiogalactopyranoside.

Mutant of fructose-1,6-bisphosphatase--A mutant of recombinant porcine liver FBPase, Gly122→Ala, was obtained by site-directed mutagenesis. A mutagenic oligonucleotide primer, 5'-CCC-CTC-GAT-GCA-TCG-TCG-AAC-3', was synthesized by the ß-cyanoethylphosphoramidite method at the Nucleic Acid Facility at Iowa State University. The codon GCA was used to mutate Gly122→Ala. BamHI/SphI fragments encoding FBPase from pEt-11a were ligated into a previously digested PUC118 plasmid. The mutagenesis was done by using single-stranded DNA from recombinant pUC118 plasmid as the template and

synthesized oligonucleotide as primer. The oligonucleotide-directed *in vitro* mutagenesis procedure was performed as described by Nakamaye and Eckstein (20). Mutagenesis was verified by dideoxy chain termination sequencing (21). The *Bam*HI/*Xba*I fragments encoding the mutations were ligated back into previously digested pEt-11a expression vector. pEt-11a was used to transform *Escherichia coli* strain BL21 (DE3).

Protein Assay--Total protein was measured by the Bio-Rad method with bovine serum albumin (from Sigma) as a standard.

Circular Dichroism Spectrometry--CD studies on the wild-type and mutant form of FBPase were carried out in 50 mM Tris-HCl buffer (pH7.5) at room temperature in an AVIV CD spectrometer model 62DS kindly supplied by Dr. Earl Stellwagen at the University of Iowa. Samples were placed in a 1-mm cuvette, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed by using a program in the computer of the spectrometer.

Kinetic Studies--FBPase activity during purification and the specific activity of pure enzyme were measured by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (22). All other kinetic experiments were done using a fluorometric assay (4) at pH 7.5 (50 mM Hepes buffer) and 24^{o}C. The initial-rate data were analyzed for kinetic mechanisms by using a Minitab language program with an α value of 2.0 (4). Cooperativity was

evaluated by using both the ENZFITTER program (23) and the Minitab program.

Results

Enzyme Quality--The purity of wild-type recombinant FBPase and the G122A mutant of porcine FBPase was evaluated by SDS-PAGE. The results are shown in Fig. 1.

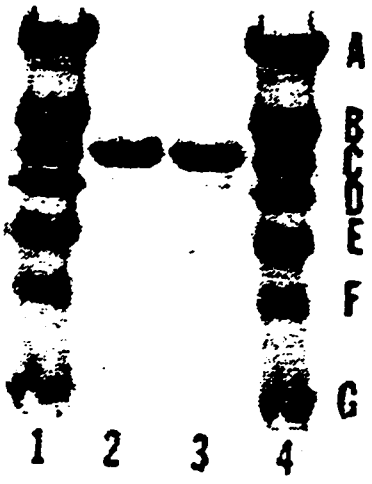


Fig.1 SDS-PAGE analysis of purified wild-type and mutant G122A porcine FBPase. All samples were run on a 12% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Lane 1, protein standard; lane 2, G122A; lane 3, wild-type. Molecular mass of protein standards: A, 66KDa; B, 45KDa; C, 36KDa; D, 29KDa; E,24KDa; F, 20KDa; G, 14KDa.

It was found that the proteins are greater than 95% pure using the criterion of electrophoresis. Also, the single band exhibiting a molecular mass of approximately 37 KDa in each lane indicates that the proteins had not undergone discernible degradation.

Secondary Structure Analysis--The secondary structures of recombinant wild-type and the G122A mutant of FBPase were analyzed by CD Spectrometry. The purpose of this study was to determine whether localized or global structural alterations were induced in the mutant. The CD spectral data showed that the spectrum of the mutant was essentially superimposable on that of the wild-type enzyme (data not shown). These results suggest that no major conformational changes occurred in FBPase when Gly122 was mutated to alanine by using CD as a criterion of secondary protein structure.

Initial Rate Studies--Table I shows the kinetic parameters for wild-type and the G122A mutant of FBPase. The data were obtained by measuring the initial rate at saturating Mg^{2+} or Fru-1,6-P_2 concentrations. From Table I, it can be seen that replacement of Gly122 with alanine caused a significant decrease in enzyme activity. The k_{cat} of the G122A mutant of FBPase was only about 5% of that of the wild-type enzyme, whereas the K_m for Fru-1,6-P_2 of this mutant form of the enzyme did not exhibit a large alteration; i.e., only a 3-fold increase compared with that of the wild-type enzyme.

Table I. Kinetic parameters for wild-type and mutant forms of FBPase

Enzyme	K_{cat} (s^{-1})	K_m Fru-1,6-P_2 (μM)	K_a[a] Mg^{2+}	Hill coef. Mg^{2+}	K_i[b] AMP $(\mu M)^2$	K_i[b] Fru-2,6-P_2 (μM)
wild-type	20 ± 0.91	2.5 ± 0.31	0.50 ± 0.04	2.0 ± 0.09	17 ± 5.4	0.36 ± 0.05
G122A	0.96 ± 0.07	8.2 ± 0.99	11 ± 2.57	1.1 ± 0.19	1370 ± 177	0.99 ± 0.29

(a) The unit of K_a for wild-type FBPase is mM^2 and for G122A it is mM.

(b) K_i values were calculated from plots of 1/velocity versus 1/Fru-1,6-P_2.

Mg^{2+} Ion Activation--The Hill coefficient and K_a for $Mg^{2+}$the wild-type and mutant form of FBPase was determined at their saturating substrate concentrations; i.e., wild-type enzyme at 12 μM and G122A at 60 μM. As expected, the Hill coefficient of wild-type FBPase for Mg^{2+} was about 2.0, and Mg^{2+} activation of FBPase was sigmoidal (data not shown). These results indicate that Mg^{2+} activation of FBPase exhibits cooperativity. This is consistent with previous reports (13-15). It was observed; however, that altering Gly122 to alanine caused the Hill coefficient for Mg^{2+} to decrease to about 1; i.e., the Mg^{2+} activation of FBPase was hyperbolic rather than sigmoidal (data not shown). These results indicate that replacement of Gly122 with alanine results in a total loss of Mg^{2+} cooperativity. Furthermore, this mutant form of FBPase exhibited a significant decrease in its affinity for Mg^{2+}; i.e., the K_a for Mg^{2+} of this mutant form of the enzyme is about 15-fold greater than that of the wild-type enzyme.

Kinetics of Fru-2,6-P_2 Inhibition--It is well known that Fru-2,6-P_2, like the substrate Fru-1,6-P_2, binds at the active site of FBPase (24-26). Since one is a substrate and the other a competitive inhibitor, FBPase must have the ability to distinguish between these two molecules. The K_i for Fru-2,6-P_2 and the K_m for Fru-1,6-P_2 of the G122A mutant did increase slightly (about 3-fold) relative to wild-type FBPase. These results indicate that the Gly122 residue is not important in

permitting FBPase to discriminate between the substrate and inhibitor. In other words, it is not directly involved in Fru-2,6-P_2 inhibition of FBPase.

The effects of Fru-2,6-P_2 inhibition on Mg^{2+} with the G122A mutant form of FBPase were also studied. The K_i increased with the increase of Mg^{2+} concentration as previously described (24). At 5 mM Mg^{2+}, the K_i for Fru-2,6-P_2 is about 22 μM.

Inhibition of AMP--AMP is known to be an allosteric inhibitor of FBPase (4,27), and the role of AMP is to remove divalent metal ions from FBPase (7). When the Gly122 residue was converted to alanine, the K_i for AMP of this mutant increased about 77-fold relative to that of the wild-type enzyme (Table I). This is a reasonable finding in light of the fact that, with this mutant, the Ka for Mg^{2+} also increased about 15-fold compared with that of the wild-type enzyme. It is believed that the AMP and metal binding sites can somehow communicate with each other, presumably through conformational changes (4,7,26). The binding of AMP to wild-type FBPase is known to exhibit cooperativity (27). The kinetic data for AMP inhibition with the two mutant forms of FBPase gave excellent fits to a cooperative model (28) in which the Hill coefficient is 2.0 (data not shown). Thus, the Gly122 residue is not directly involved in the cooperativity of AMP binding to FBPase.

Kinetic Studies in the Absence of Inhibitors--To confirm the finding that altering Gly122 to alanine results in the total loss of cooperativity for Mg^{2+} with FBPase, we studied the kinetics of the FBPase reaction in the absence of inhibitors. Fig. 2 shows the double-reciprocal plot of initial velocity against the concentration of Mg^{2+} at different fixed levels of Fru-1,6-P_2. When Fru-1,6-P_2 concentration was varied at different fixed concentrations of Mg^{2+}, a family of lines intersecting to the left of the 1/velocity axis was obtained for double reciprocal plots (data not shown). The data in Fig. 2 gave excellent fits to Eq. 1 where n = 1 but fit poorly when n = 2. The form of Eq. 1 is:

$$\frac{1}{V}=\frac{1}{V_m}\left[1+\frac{K_a}{A^n}+\frac{K_b}{B}+\frac{K_{ia}}{A^n}\frac{K_b}{B}\right]$$

where V, V_m, A, B, K_a, K_b, and K_{ia} represent the initial velocity, maximum velocity, the concentration of free Mg^{2+}, the concentration of free Fru-1,6-P_2, the Michaelis constant for Mg^{2+}, the Michaelis constant for Fru-1,6-P_2, and the dissociation constant for Mg^{2+}, respectively; n represents the Hill coefficient for Mg^{2+} with FBPase. When n = 1, there is no cooperativity; when n = 2, the binding of Mg^{2+} to FBPase is cooperative, with a Hill coefficient of 2. Equation 1 is the fundamental initial rate equation for the sequential kinetic mechanism shown in Scheme 1.

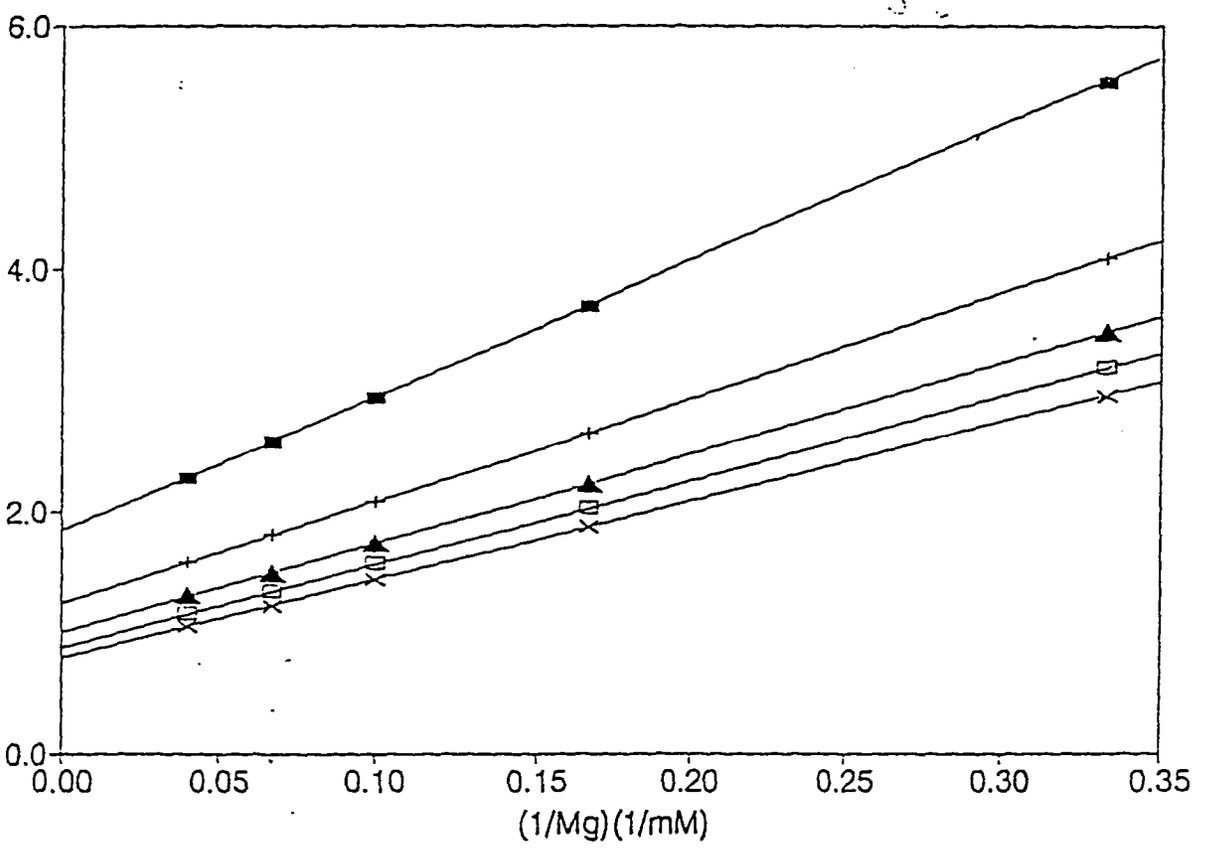


Fig.2 Plot of reciprocal of initial velocity in arbitrary fluorescent units against reciprocal of Mg^{2+} concentration for G122A FBPase. The concentrations of Fru-1,6-P_2 are 25 μM (x), 15 μM (□), 10 μM (▲), 6 μM (+), and 3 μM (■). The lines are theoretical based on Eq. 2 when n = 1, and the points are experimentally determined.

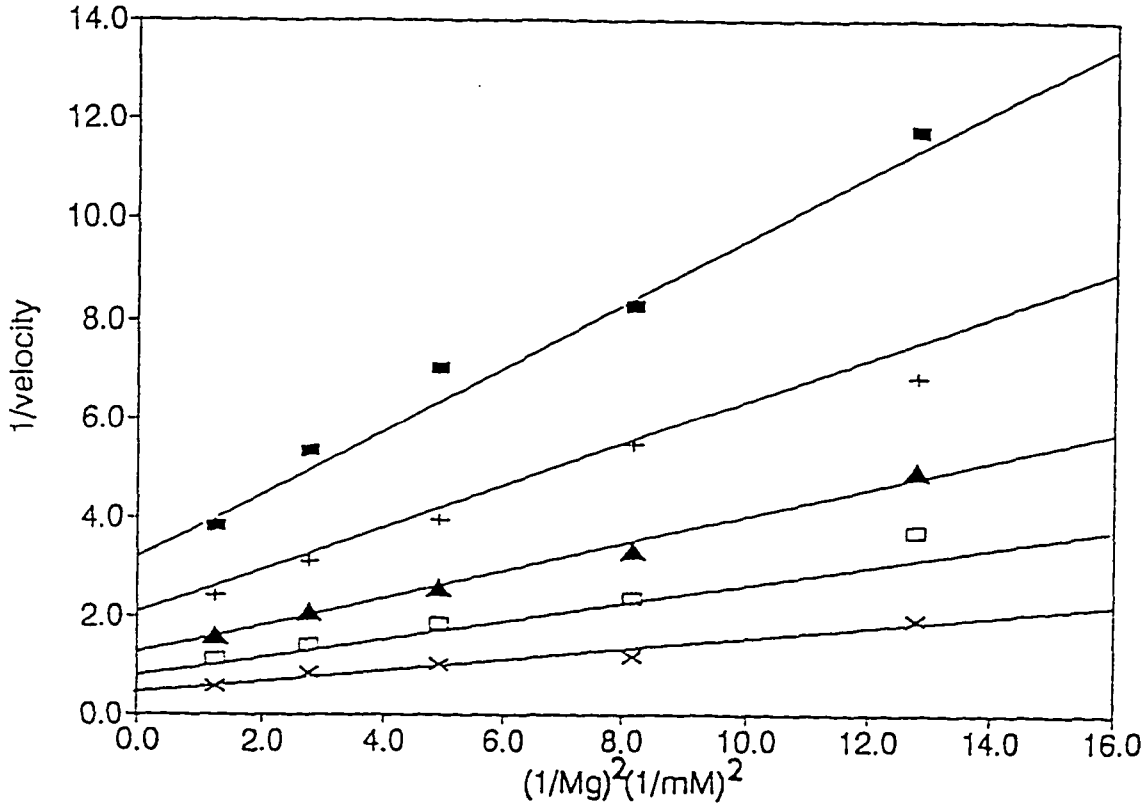
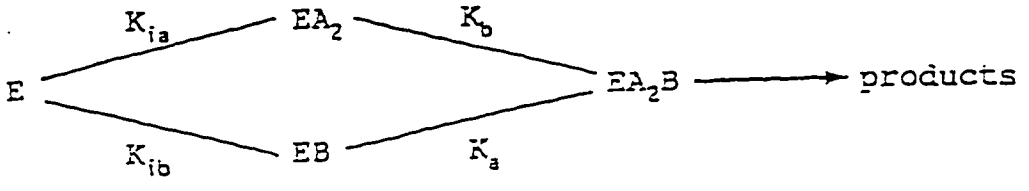


Fig.3 Plot of reciprocal of initial velocity in arbitrary fluorescent units versus reciprocal of $[Mg^{2+}]^2$ for wild-type FBPase. The concentrations of Fru-1,6-P_2 are 9.0 μM (x), 5.0 μM (□), 3.0 μM (▲), 2.0 μM (÷), and 1.2 μM (■), The lines are theoretical based on Eq. 2 when n = 2, and the points are experimentally determined.



Scheme 1

$$E + 2A = EA_2,\ K_{ia} = (E)(A)^2/EA_2; \quad (2)$$

$$E + B = EB,\ K_{ib} = (E)(B)/EB; \quad (3)$$

$$EA_2 + B = EA_2B,\ K_b = (EA_2)(B)/EA_2B; \quad (4)$$

$$EB + 2A = EA_2B,\ K_a = (EB)(A)^2/EA_2B \quad (5)$$

On the other hand, the kinetic data in the absence of inhibitors with wild-type FBPase (shown in Fig. 3) gave excellent fits to Eq. 1 when n = 2 and did not fit well to Eq. 1 when n = 1. Note that the only difference in the rate equations between the G122A mutant enzyme and the wild-type enzyme is the term n. The data shown in Fig. 3 are consistent with previous reports that show that binding of divalent metal ions to FBPase exhibits cooperativity (11,13-15), whereas the findings illustrated in Fig. 2 exclude the possibility of cooperativity for Mg^{2+} with the G122A mutant enzyme. Had Mg^{2+} ions bound cooperatively to FBPase, the data would have fit to Eq. 1 much better when n = 2 than when n = 1. On the basis of these results and the Hill coefficient obtained for G122A, it is reasonable to conclude that the Gly122 residue is required for the cooperativity observed for Mg^{2+} with FBPase.

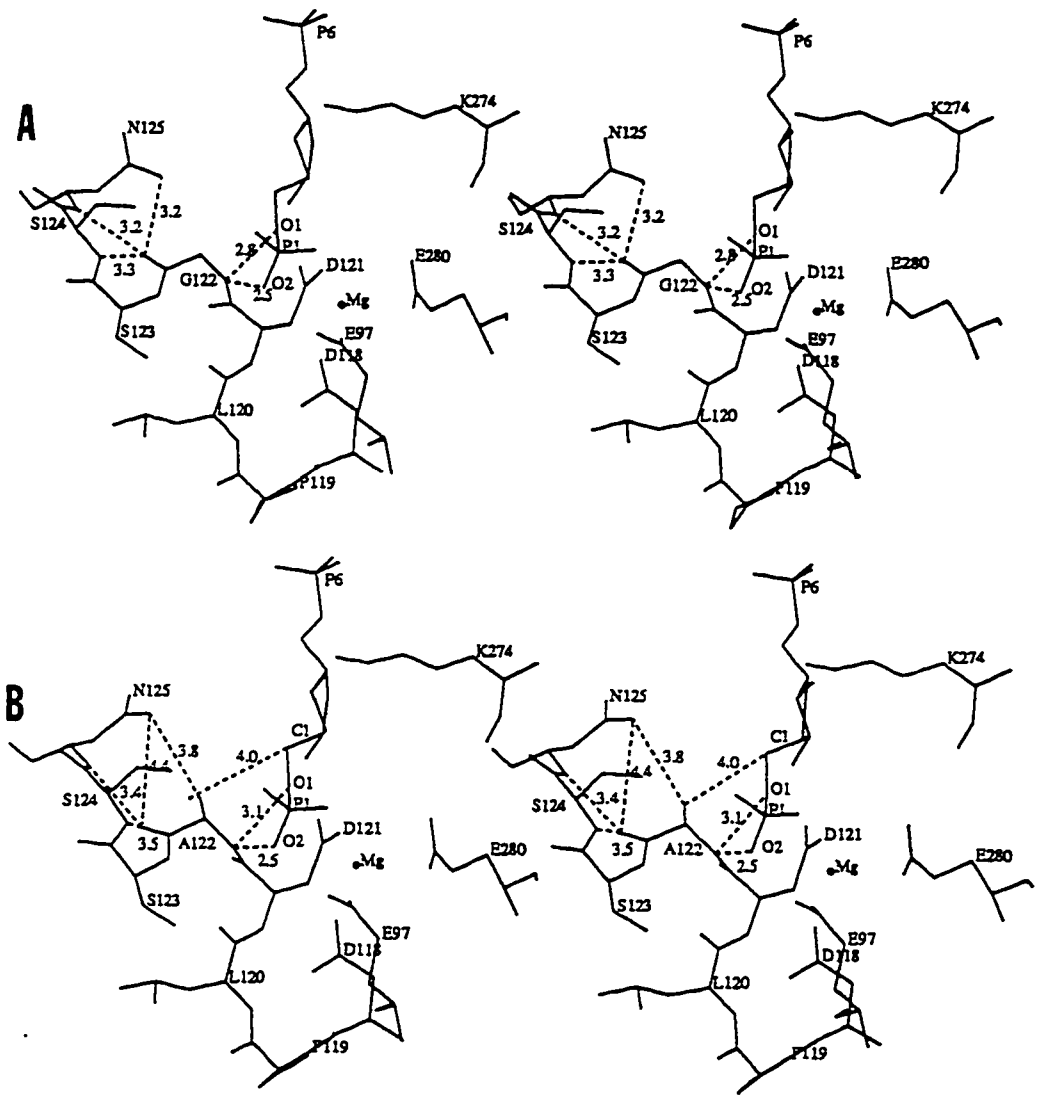
Discussion

The central finding of this report involves the complete loss of cooperativity for Mg^{2+} when FBPase Gly122 is mutated to alanine. It is known that mammalian FBPase is a divalent metal requiring enzyme (8) and that the Hill coefficient is 2.0 at neutral pH (13,14) but only 1.0 at alkaline pH (4,13,14). Crystallographic data (10) suggest that two metal ions such as Mn^{2+} and Zn^{2+} are associated with the enzyme, however, with Mg^{2+}, only one metal ion was found to bind FBPase.

Replacement of Gly122 with alanine caused a 19-fold decrease in enzyme activity and a significant decrease of the enzyme's affinity for Mg^{2+}. This is expected because the main chain NH group of Gly122 is thought to act as a donor to form a bifurcated hydrogen bond with the O1 oxygen of the fructofuranoside ring and an oxygen of the 1-phosphoryl group of the substrate (10). Therefore, this bifurcated hydrogen bond is believed to anchor the 1-phosphoryl group in the required position for both metal-binding and catalysis (10). In addition, the bifurcated hydrogen bond may contribute to catalysis by making the phosphoester bond weaker such that nucleophilic attack by OH^- on the 1-phosphorous atom would be facilitated.

A significant finding associated with this report is that the Hill coefficient of the G122A mutant FBPase for Mg^{2+} is about 1.0. This conclusion is alluded to from kinetic data

Fig.4 A). Stereo model of the active site of FBPase as defined in the crystallographic study of porcine FBPase complexed with the substrate analogue α-methyl-Fru-1,6-P_2 and Mg^{2+} (10). For clarity the binding residues of the 6-phosphate group have been omitted. The backbone NH of Gly122 is hydrogen bonded to oxygens O1 and O2 of the 1-phosphate group (2.8 Å and 2.5 Å, respectively). The backbone CO of Gly122 interacts with the backbone NH of Ser124 and Asn125, and also with the side chain NH of Asn125. The Mg^{2+} ion is coordinated to the O2 oxygen of the 1-phosphate group and to residues Glu97, Asp118 and Glu280.
B). Stereo model of the catalytic site showing the effect of the alanine substitution at position 122. If the position of backbone atoms are kept as in the cryatallographic complex shown in Fig.4A, the β-methyl group of Ala122 is only 3.4 Å from the C1 carbon of α-methyl-Fru-1,6-P_2 and only 2.4 Å from the side chain of Asn125. Slightly different positions of residues 122-125 are required in order to accommodate the presence of a β-methyl group in residue 122. The energy minimized model (Fig.4B) shows that interactions of the backbone NH of Ala122 with the oxygen O1 and O2 of the 1-phosphate group of the substrate analog are maintained. The β-methyl group of Ala122 is 4 Å from the C1 carbon of the substrate analog and 3.8 Å from the side chain of Asn125.


A
P6
K274
N125
S124
3.2
3.2
O1
P1
2.8
3.3
G122
3.5
O2
D121
Mg
E280
S123
E97
D118
L120
P119
B
P6
K274
N125
3.8
C1
4.0
S124
3.4
O1
P1
3.1
3.5
A122
O2
2.5
D121
Mg
E280
S123
E97
D118
L120
P119

obtained in the absence of inhibitors. These results suggest that altering Gly122 of FBPase to alanine essentially causes the complete loss of Mg^{2+} cooperativity. Furthermore, the affinity of this mutant form of FBPase for metal ions decreased markedly. From these results, we conclude that the Gly122 residue of mammalian FBPase is essential for both cooperativity and binding of Mg^{2+} to the enzyme. An understanding of this important finding requires further investigations (e.g., solution of the crystal structure of G122A FBPase); however, modeling studies were undertaken to provide some insight into the effect of substituting alanine for glycine at position 122. Gly122 is located in a three residue loop between strand B3 (residues 113-118) and helix H4 (residue 123-127). If one compares the various crystallographic complexes of FBPase, it is obvious that residues 121-127 are subject to conformational changes during catalysis (27): root mean square (rms) deviation for these residues range between 0.29 and 0.70 Å (between native FBPase and FBPase complexed with Fru-1,6-P_2, with α-methyl-Fru-1,6-P_2, or with Fru-6-P, respectively). Most interestingly, Gly122 is at a hinge point between strand B3 and helix H4.

The active site region of FBPase, based on the crystal structure of the enzyme (10), is shown in Fig.4. If Gly122 (Fig.4A) is replaced by Ala122 (Fig.4B), the ß-methyl group is located in the Fru-1,6-P_2 binding site, close to the C1 carbon of the substrate or its analog. Ala122 can reduce the

flexibility of the 122-127 region or lock the enzyme in a conformation which suppresses cooperativity for Mg^{2+}. In addition, binding of Mg^{2+} causes repositioning of the 1-phosphoryl group of Fru-1,6-P_2 or its analog, α-methyl Fru-1,6-P_2 (27). Based on these structural studies, the following events can be proposed: when the substrate binds, the 1-phosphoryl group adopts an initial conformation. The binding of Mg^{2+} in the metal site moves the 1-phosphoryl group into a position that allows cleavage of the P-O bond, yielding the formation of Fru-6-P. The ß-methyl group of Ala122 can lock the 1-phosphoryl group in a defined conformation when substrate binds and this affects the hinge movement of helix H4, disrupting some interactions that are required for signal transmission between monomers.

It is well documented that the mechanism of regulation of FBPase involves AMP and Fru-2,6-P_2, which are potent synergistic inhibitors of the enzyme (28-31). Fromm and coworkers (4,7,25) have shown that AMP and metal ions are mutually exclusive in their binding to the enzyme. X-ray diffraction studies suggested that the metal binding sites are far from the allosteric sites for AMP (32). The data shown in Table I indicate that the K_i for AMP of the G122A mutant is much higher than that of the wild-type enzyme. It is obvious that converting Gly122 to alanine, not only affects the enzyme's affinity for divalent metal ions, but also affects the enzyme's affinity for AMP. These results are consistent

with previous suggestions that the metal binding sites and the allosteric sites for AMP can somehow communicate with each other (4,7,28). When AMP binds to the enzyme, the coordination sphere of Mg^{2+} is affected as follows: side chains of residues Glu97, Asp118 and Asp121 belonging to strand B3 move from their R state conformation 1.79 Å (OE1), 1.71 Å (OD2) and 4.10 Å (OD1). Residues Lys274 and Glu280 are also affected upon binding (27). From our results, Gly122 located in the hinge region between strand B3 and helix H4, seems to be directly involved in the signal transmission pathway for communication between metal binding sites and allosteric sites.

The binding of AMP to wild-type FBPase exhibits cooperativity (28). Site-directed mutagenesis at the AMP binding site led to a total loss of cooperativity for AMP with FBPase (28). The kinetic data of AMP inhibition with G122A FBPase gave excellent fits to a cooperative model. This finding suggests that the Gly122 residue may not be involved in the cooperativity of AMP, although it is absolutely required for Mg^{2+} cooperativity.

It is believed that Fru-2,6-P_2 has two functions in regulating FBPase. One is that it is a potent competitive inhibitor of the substrate and competes with Fru-1,6-P_2 at the enzyme's active site (24,25,31,33). The second function is to enhance the effect of AMP by making AMP "stickier" to the enzyme (7). AMP is known to be a potent competitive inhibitor

of Mg^{2+} (28) and to prevent divalent metal ion binding to the enzyme; i.e., the two ligands are mutually exclusive in their binding to FBPase.

The findings of this report demonstrate that Gly122 is essential for the well-established cooperativity and divalent metal binding affinity of porcine liver FBPase. The x-ray diffraction investigations of FBPase by the Lipscomb group (34) has provided a basis for AMP cooperativity. The enzyme functions as a dimmer of dimmers in which R and T states exist. In the case of AMP binding, the T state is induced, whereas the substrate adds to the R state of the enzyme. This model provides a rational explanation for a Hill coefficient of 2 for AMP. On the other hand, an explanation of divalent metal ion binding seems to be less well understood. Two binding sites for either Mn^{2+} or Zn^{2+} have been demonstrated; however, the x-ray diffraction studies can pinpoint only a single Mg^{2+} ion per monomer. In addition, kinetic studies at pH 9.6 (4) accord with the latter finding; i.e., only 1 Mg^{2+} per monomer and no cooperativity. It is well established that at neutral pH, Mg^{2+} binding is cooperative and the Hill coefficient is 2 (11,13-15). The data shown in Fig. 3 confirm this finding. The origin of Mg^{2+} binding cooperativity remains unclear even though the metal binding sites of FBPase were markedly altered by site-directed mutagenesis (16). Although the k_{cat} of the mutant FBPase was decreased by at least three orders of magnitude relative to the wild-type

enzyme, the cooperativity of metal ions did not show significant alteration (16). These results suggest, but do not provide conclusive proof, that divalent metal ion cooperativity is inter- rather than intra-subunit. The results of the present study may lead to a better understanding of the basis of the cooperativity phenomenon for metal ion binding to FBPase at the molecular level when the three-dimensional structure of the Gly122 mutant becomes available.

References

1. Underwood,A.H., and Newsholme,E.A. (1965) Biochem. J. **95**, 767-774.
2. McGilvay,R.W. (1964) Fructose-1,6-diphosphatase and its role in Gluconeogenesis (McGilvay, R.W., and Pogell, B.M.,Eds). Port City Press Inc, Baltimore.
3. Krebs,H.A. (1963) Advances in Enzyme Regulation, Vol.1, (Weber, G., Ed.) Pergamon Press Ltd., London and New York.
4. Liu,F., and Fromm,H.J.(1990) J. Biol. Chem. **265**, 7401-7406.
5. Hers,H.-G., and Van Schaffingen,E.(1982)Biochem. J. **206**, 1-12.
6. Scheffler,J.E., and Fromm,H.J.(1986)Biochemistry **25**, 6659-6665.
7. Liu,F., and Fromm,H.J.(1988)J. Biol. Chem. **263**,9122-9128.
8. Gomori,G.(1943) J. Biol. Chem. **148**, 139-149.
9. Hubert,E., Villanueva,J., Gonzalez,A.M., and Marcus,F.

(1970) Arch. Biochem. Biophys. **138**, 590-597.

10. Zhang,Y., Liang,J.-Y., Huang,S., Ke, H., and Lipscomb,W.N. (1993) Biochemistry **32**, 1844-1857.
11. Benkovic,S.J., and deMaine, M.M. (1982) Adv. Enzymol. Relat. Areas Mol. Biol. **53**, 45-82.
12. Benkovic,P.A., Caperelli,C.A., deMaine,M.M., and Benkovic, S.J. (1978) Proc. Natl. Acad. Sci. U.S.A., **75**, 2185-2189.
13. Nimmo,H.G., and Tipton,K.F. (1975) Eur. J. Biochem. **58**, 567-574.
14. Nimmo,H.G., and Tipton,K.F. (1975) Eur. J. Biochem. **58**, 575-585.
15. Tejwani, G. (1983) Adv. Enzymol. Relat. Areas Mol. Bio. **54**, 121-193.
16. Chen,L., Remesh,H., Chen,M., and Fromm,H.J. (1993) Arch. Biochem. and Biophys. **307**, 350-354.
17. El-Maghrabi, M.R., Gidh-Jain,M., Austin,L.R., and Pilkis, S.J. (1993) J. Biol. chem., **268**, 9466-9472.
18. Marcus,F., Edelstein,I., Reardon,I., and Heinrikson,R.L. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 7161-7165.
19. Burton, V.A., Chen,M., Ong,W.C., Ling,T., Fromm,H.J., and Stayton,M.M. (1993) Biochem. Biophys. Res. Commun. **192**, 511-517.
20. Nakamaye, K., and Eckstein,F. (1986) Nucleic Acids Res. **14**, 9679-9898.
21. Sanger,F., Nicklen,S., and Coulson,A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463-5467.

22. Pontremodi, S., and Traniello, S. (1975) Methods Enzymol. **42**,369-374.

23. Leatherbarrow,R.J. (1987) Enzfitter, A Non-Linear Regression Data Analysis Program for the IBMPC, pp.13-75, Elsevier Science Publishers BV, Cambridge, United Kingdom.

24. Ke,H., Thorpe,C.M., Seaton,B.A., Marcus,F., and Lipcomb,W.N. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 1475-1479.

25. Ke,H., Thorpe,C.M., Seaton,B.A., Lipscomb,W.N., and Marcus,F. (1990) J. Mol. Biol. **212**, 513-539.

26. Scheffer,J.E., and Fromm,H.J. (1986) Biochemistry **25**, 6659-6665.

27. Liang, J.-Y., Zhang, Y., Huang, S., Ke, H., and Lipscomb, W.N. (1992) "Regulation of Protein by Ligands". Proc. Robert A. Welch Foundation Conf. Chem. Res., October 26-27, Houston, Texas.

28 Chen,M., Chen,L., and Fromm,H.J. (1994) J. Biol. Chem. **269**, 5554-5558.

29. Van Schaffingen,E., Hue,L., and Hers,H.G. (1980) Biochem. J. **192**, 887-895.

30. Van Schaffingen,E., Hue,L., and Hers,H.G. (1980) Biochem. J. **192**, 897-901.

31. Van Schaffingen,E., and Hers,H.G. (1980) Biochem. Biophys. Res. Commun., **96**, 1524-1531.

32. Pilkis,S.J., EL-Maghrabi,M.R., Pilkis,J., and Claus,T. (1981) J. Biol. Chem. **256**, 3619-3622.

33. Ke,H., Zhang,Y., and Lipscomb,W.N. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5243-5247.

34. Ganson,N.J., and Fromm,H.J (1982) Biochem. Biophys. Res. Commun. **108**, 233-239.

35. Ke,H., Liang,J.Y., Zhang,Y., and Lipscomb,W.N. (1991) Biochemistry **30**, 4412-4420.

CHAPTER 3. MUTATION OF ARGININE 276 TO METHIONINE CHANGES Mg^{2+} COOPERATIVITY AND THE KINETIC MECHANISM OF FRUCTOSE-1,6-BISPHOSPHATASE[1]

A paper published in Journal of Biological Chemistry[2]

Rulin Zhang[3,4] and Herbert J. Fromm[3,5]

Abstract

Arginine 276 of porcine liver fructose-1,6-bisphosphatase (FBPase) was mutated to methionine by site-directed mutagenesis based on the crystal structure of the enzyme [Zhang, Y., Liang, J.-Y., Huang, S., Ke, H., and Lipscomb, W.N. (1993) Biochemistry 32,1844-1857]. The mutant and wild-type forms of the enzyme were purified to homogeneity and characterized by circular dichroism spectrometry (CD) and initial-rate kinetics. There were no discernible differences between the secondary structures of wild-type and the mutant enzymes on the basis of the CD data. Replacing Arg 276 with methionine caused a significant decrease in the enzyme's

---

[1]This research was supported in part by Research Grants NS 10546 and GM06920 from the National Institutes of Health, United States Public Health Service, and Grant MCB-9218763 from the National Science Foundation. This is Journal Paper J-16229 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Project 2575.

[2]Reprinted with permission of Biochemistry, 1996,34, 8190-8195.

[3]Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011.

[4]Primary researcher and author under the supervision of Herbert J. Fromm.

[5]To whom correspond should be addressed.

activity. The k_{cat} for this mutant enzyme was only about 0.67% of that of the wild-type enzyme. Most importantly, the mutation caused the total loss of cooperativity for Mg^{2+} and changed the kinetic mechanism to one in which the substrate adds to FBPase before Mg^{2+} and in which all steps equilibrate rapidly relative to the conversion of the ternary complex of enzyme, substrate, and Mg^{2+} to products. The K_a for Mg^{2+} increased only about 5-fold relative to that of the wild-type enzyme. The mutation did not change the K_i for AMP and the Hill coefficient of this allosteric inhibitor. The K_i for fructose 2,6-bisphosphate was increased 16-fold compared with that of the wild-type enzyme. The K_m for fructose 1,6-bisphosphate was similar to that of wild-type enzyme. It is concluded that Arg 276 is critical for activity and Mg^{2+} cooperativity with FBPase, and it determines the enzyme's kinetic mechanism.

Introduction

Fructose 1,6-bisphosphatase (FBPase, EC3.1.3.11) is located at a key regulatory position in the gluconeogenic pathway (Krebs, 1963; Marcus, 1981; Pilkis et al., 1988; Hers and Hue, 1983). In the presence of a divalent metal ion, it catalyzes the hydrolysis of fructose 1,6-bisphosphate (Fru-1,6-P_2) to form fructose 6-phosphate (Fru-6-P) and inorganic phosphate (P_i) and exhibits a rapid-equilibrium random Bi Bi kinetic mechanism (Liu and Fromm, 1990). The reaction is

competitively inhibited by fructose 2,6-bisphosphate (Fru-2,6-P_2) and noncompetitively inhibited by adenosine 5'-monophosphate (AMP) (Liu and Fromm, 1990). Both compounds act synergistically to inhibit FBPase (Hers and Van Schaftingen, 1982). It has been shown from both binding (Scheffer and Fromm, 1986) and kinetic studies (Liu and Fromm, 1990) that Mg^{2+} and AMP are mutually exclusive binding ligands. It is thought that one of the functions of Fru-2,6-P_2 is to make AMP stickier on the enzyme, thus enhancing the action of AMP (Liu and Fromm, 1988).

It is well known that porcine kidney FBPase is a homotetramer with a subunit molecular weight of 37 KDa (Marcus et al., 1982; Burton et al., 1993), and like all FBPases, it requires a divalent metal ion for activity (Gomori, 1943; Benkovic and deMaine, 1982). It has been shown from binding investigations that there are two metal-binding sites per enzyme subunit: a high affinity "structural" site and a low affinity "catalytic" site (Benkovic et al., 1978), and catalysis requires the sequential addition of metal, substrate, and catalytic metal to form a catalytically competent quaternary complex of enzyme-M1-M2-Fru-1,6-P_2. Nimmo and Tipton (1975a,b) showed that pH could change the Mg^{2+} kinetics of bovine liver FBPase. At neutral pH, Mg^{2+} binding and activation of bovine liver FBPase shows cooperativity, but the cooperativity is lost at pH9.6. Chen et al.(1993) and El-Maghrabi et al.(1993) did site-directed

mutagenesis in the metal binding sites of mammalian FBPase. Their results support the suggestion that there are two metal binding sites associated with FBPase, but the Mg^{2+} cooperativity is inter- rather than intra-subunit. Zhang et al.(1995) showed that porcine liver FPBase lost Mg^{2+} cooperativity at pH 7.5 when Gly 122 was mutated to alanine.

X-ray diffraction results (Zhang et al., 1993) of porcine kidney FBPase showed that the side chain ε NH_2 of Arg 276 in the C1 chain formes a salt bridge with the 1-phosphate oxygen O11 at the α anomer of the substrate. But in the presence of metal ions, the side chain of Arg 276 no longer interacts with the 1-phosphate oxygen atom. This occurs because the binding of metal ions causes slight changes of orientation and position of the sugar ring and moves the 1-phosphoryl group of the substrate toward the metal binding sites. Thus, the residue is postulated to play roles in enzyme catalysis and binding of metal ions. To verify this postulation, we altered Arg 276 of porcine liver FBPase to methionine by site-directed mutagenesis and have studied its properties. In this study, we report that replacement of Arg 276 with methionine changes the Mg^{2+} cooperativity and the kinetic mechanism of porcine liver FBPase and causes a significant decrease in the enzyme's activity.

Experimental Procedures

Materials--NADP, fructose 1,6-bisphosphate (Fru-1,6-P_2), fructose 2,6-bisphosphate (Fru-2,6-P_2), AMP, Hepes, and Tris were purchased from Sigma (St. Louis, MO). Glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim (Indianapolis, IN). Distilled deionized water was used in all experiments. All other reagents were of the highest purity available commercially. Recombinant and mutant forms of porcine liver FBPase were prepared and purified as described elsewhere (Burton et al., 1993) with slight modifications. Mutant forms of the enzyme were obtained in yields comparable to the wild-type enzyme. Porcine liver and kidney FBPase are identical in their primary sequences (Burton et al., 1993).

Mutant of fructose-1,6-bisphosphatase--A mutant of recombinant porcine liver FBPase, R276M, was obtained by site-directed mutagenesis. A mutagenic oligonucleotide primer, 5'-GGA-AAG-TTA-**ATG**-CTG-CTA-TAC-3', was synthesized by the ß-cyanoethylphosphoramidite method at the Nucleic Acid Facility at Iowa State University. The codon ATG was used to mutate Arg276→Met. BamHI/SphI fragments encoding FBPase from pEt-11a were ligated into a previously digested PUC118 plasmid. The mutagenesis was done by using single-stranded DNA from recombinant pUC118 plasmid as the template and synthesized oligonucleotide as primer. The oligonucleotide-directed in vitro mutagenesis procedure was performed as described by

Nakamaye and Eckstein (1986). Mutagenesis was verified by dideoxy chain termination sequencing (Sanger et al., 1977). The BamHI/XbaI fragments encoding the mutations were ligated back into previously digested pEt-11a expression vector. pEt-11a was used to transform Escherichia coli strain BL21 (DE3).

Protein Assay--Total protein was measured by the Bio-Rad method with bovine serum albumin (from Sigma) as a standard.

Circular Dichroism Spectrometry--Circular dichroism spectroscopic (CD) studies on the wild-type and mutant form of FBPase were carried out in 5 mM Hepes buffer (pH7.5) at room temperature in a JASCO CD spectrometer model J-710. Samples were placed in a 1-mm cuvette, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed by using a program in the computer of the spectrometer.

Kinetic Studies--FBPase activity during purification and the specific activity of pure enzyme were measured by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (Pontremoli and Traniello, 1975). All other kinetic experiments were done using a fluorometric assay (Liu and Fromm, 1990) at pH 7.5 (50 mM Hepes buffer) and 24^{o}C. The initial-rate data were analyzed for kinetic mechanisms by using a MINITAB language program with an α value of 2.0 (Liu and Fromm, 1990). Cooperativity was evaluated by using both the ENZFITTER program (Leatherbarrow, 1987) and the MINITAB program.

Results

Enzyme Quality--Wild-type recombinant porcine liver FBPase and the R276M mutant FBPase were purified to greater than 95% pure by using electrophoresis as a criterion (data not shown).

Secondary Structure Analysis--The secondary structures of both recombinant wild-type and R276M mutant forms of FBPase were analyzed by CD spectrometry. The CD spectral data showed that the spectrum of the mutant enzyme was completely superimposable on that of the wild-type enzyme (data not shown). These results indicate that no major conformational changes occurred with FBPase when Arg 276 was mutated to methionine.

Initial-Rate Kinetics--To evaluate the effects of the mutation on the properties of FBPase, kinetic studies were carried out for the mutant and wild-type forms of FBPase at either saturating Mg^{2+} or Fru-1,6-P_2 concentrations. The kinetic parameters are summarized in Table 1. From Table 1, it can be seen that replacement of Arg 276 with methionine caused a significant decrease in the enzyme's activity. The k_{cat} for this mutant enzyme was only about 0.67% of that of the wild-type enzyme, whereas the K_m for Fru-1,6-P_2 was similar to that of the wild-type enzyme.

Table 1

Kinetic parameters for wild-type and mutant forms of fructose-1,6-bisphosphatase

Enzyme	k_{cat} (s^{-1})	K_m Fru-1,6-P_2 (μM)	K_a [a]	Mg^{2+} Hill coef.	K_i [b] AMP (μM)^2	K_i [b] Fru-2,6-P_2 (μM)
wild-type	20 ± 0.91	2.5 ± 0.31	0.50 ± 0.04	2.04 ± 0.09	17.8 ± 5.4	0.36 ± 0.05
R276M	0.13 ± 0.0	3.1 ± 0.34	2.8 ± 0.32	1.18 ± 0.13	37.8 ± 6.7	5.8 ± 0.54

[a] The unit for K_a for wild-type FBPase is mM^2, and for R276M, it is mM.

[b] K_i values were determined at 5 mM and 8 mM Mg^{2+} for wild-type and R276M mutant respectively.

Mg^{2+} Ion Activation--As expected, the Hill coefficient of wild-type FBPase for Mg^{2+} was 2.0, and Mg^{2+} activation of the enzyme was sigmoidal (data not shown). These results indicate that Mg^{2+} activation of FBPase exhibits cooperativity. This is consistent with previous reports (Nimmo and Tipton, 1975a,b). However, it was found that the Hill coefficient of the mutant FBPase for Mg^{2+} was about 1; i.e., Mg^{2+} activation of FBPase was hyperbolic instead of sigmoidal (data not shown). In these studies, the level of Mg^{2+} was varied 120-fold (0.1-12 mM) at a saturating concentration of Fru-1,6-P_2. No evidence of cooperativity, either positive or negative, was observed. These results indicate that the mutation of Arg276 to methionine caused a total loss of Mg^{2+} cooperativity, although the K_a for Mg^{2+} increased only about 5-fold relative to that of wild-type FBPase.

Inhibition by AMP--AMP is an allosteric inhibitor of FBPase (Nimmo and Tipton, 1975a; Taketa and Pogell, 1965; Stone and Fromm, 1980), and the function of AMP is to remove divalent metal ion from FBPase (Liu and Fromm, 1988). AMP binding to FBPase shows cooperativity (Chen et al., 1994). When Arg 276 was mutated to methionine, the K_i for AMP increased only about 2-fold (Table 1) relative to the wild-type enzyme, and the AMP inhibition pattern still exhibited cooperativity. These data suggest that Arg 276 is not involved in AMP inhibition and cooperativity.

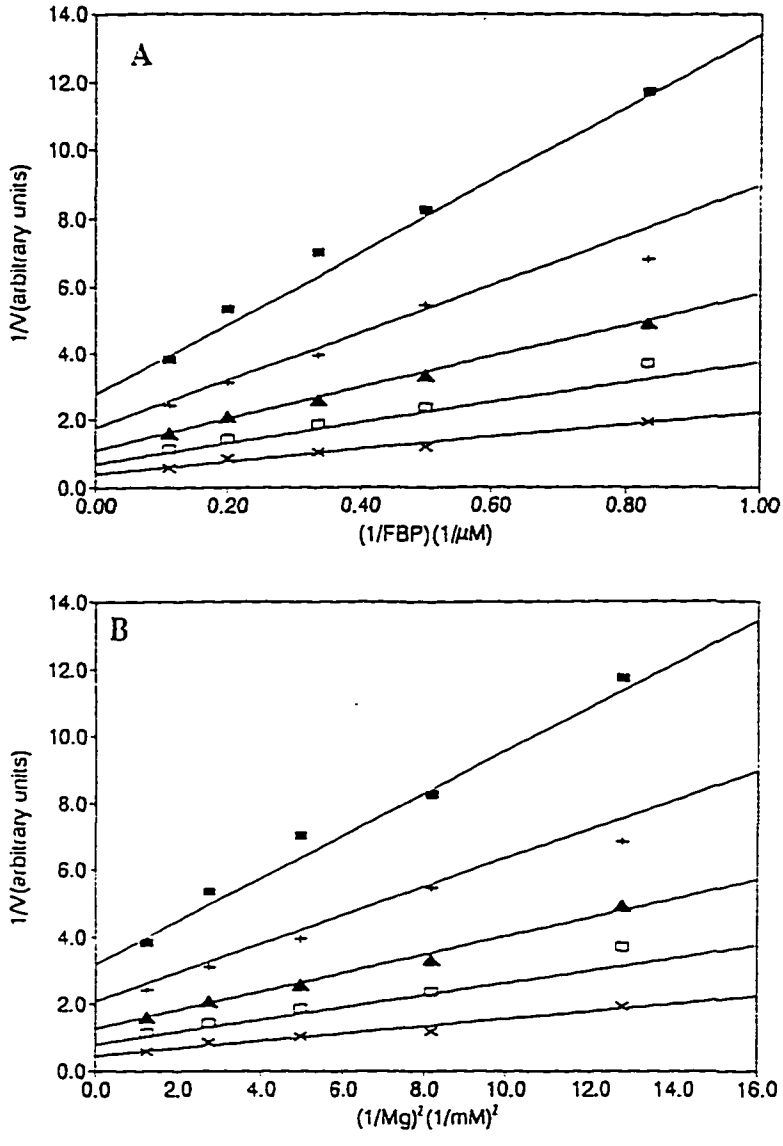


Fig.2 A) Plot of reciprocal of initial velocity with wild-type FBPase in arbitrary fluorescent units versus reciprocal of $[Fru\text{-}1,6\text{-}P_2]$ concentrations. The concentrations of Mg^{2+} are 0.28 mM (■), 0.35 mM (+), 0.45 mM (▲), 0.60 mM (□), and 0.90 mM (x). The lines are theoretical based on Equation 1 when n = 2, and the points are experimentally determined.

Fig.2B Plot of reciprocal of initial velocity with wild-type FBPase in arbitrary fluorescent units against reciprocal of $[Mg^{2+}]^2$ concentrations. The concentrations of $[Fru\text{-}1,6\text{-}P_2]$ are 1.2 μM (■), 2.0 μM (+), 3.0 μM (▲), 5.0 μM (□), and 9.0 μM (x). The lines are theoretical based on Equation 1 when n = 2, and the points are experimentally determined.

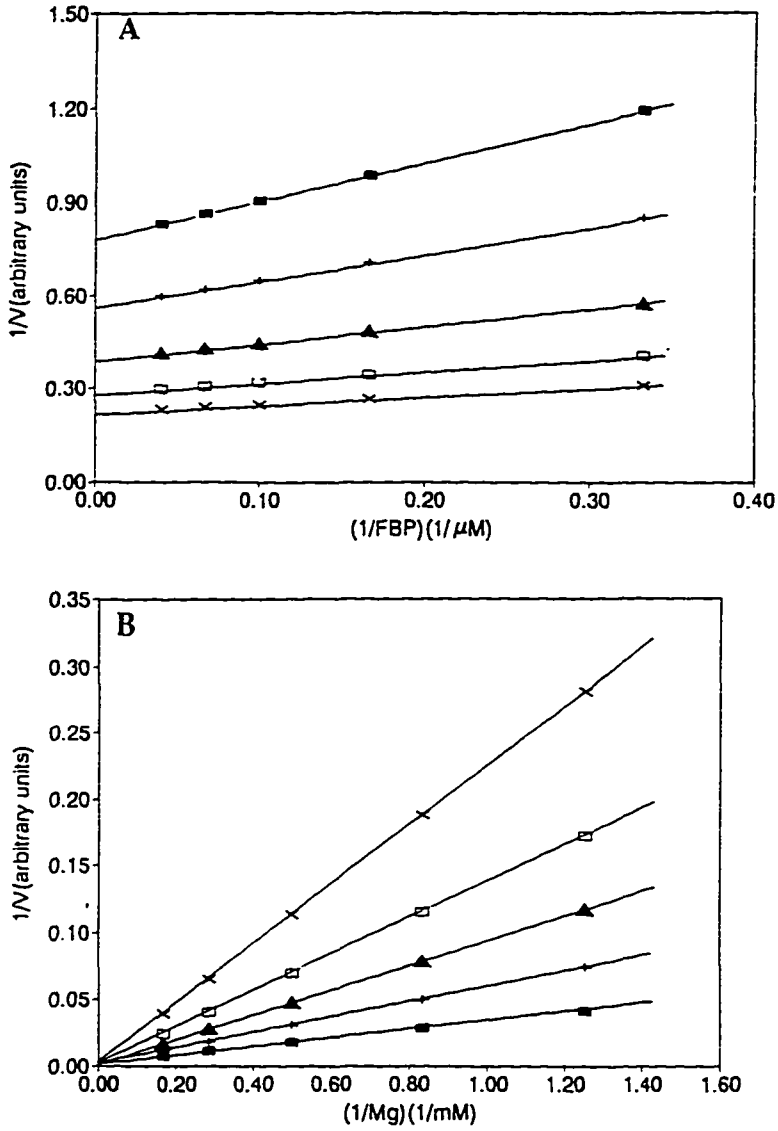


Fig. 3A Plot of reciprocal of initial velocity with R276M mutant FBPase in arbitrary fluorescent units versus reciprocal of [Fru-1,6-P_2] concentrations. The concentrations of Mg^{2+} are 0.8 mM (■), 1.2 mM (+), 2.0 mM (▲), 3.5 mM (□), 6.0 mM (x). The lines are theoretical based on Equation 1 when n = 1, and the points are experimentally determined.

Fig. 3B Plot of reciprocal of initial velocity with R276M mutant FBPase in arbitrary fluorescent units versus reciprocal of [Mg^{2+}] concentrations. The concentrations of [Fru-1,6-P_2] are 25 μM (■), 15 μM (÷), 10 μM (▲), 6 μM (□), and 3 μM (x). The lines are theoretical based on Equation 1 when n = 1, and the points are experimentally determined.

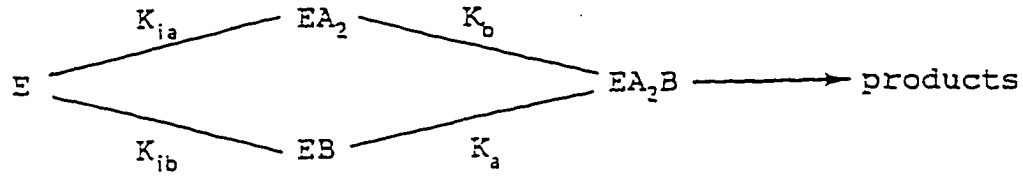
Inhibition by Fru-2,6-P_2--Fru-2,6-P_2 is a competitive inhibitor of Fru-1,6-P_2 and competes with the substrate for the active site of FBPase (Pilkis et al., 1981; Pontremoli et al., 1982; Ganson and Fromm, 1982; McGrane et al., 1982; Liu and Fromm, 1989; Ke et al., 1989, 1990; Scheffler and Fromm, 1986). From Table 1, it can be seen that the K_i of the R276M mutant FBPase for Fru-2,6-P_2 increased about 16-fold relative to that of wild-type FBPase. These results indicate that Arg 276 is not as important as Lys 274 in permitting FBPase to discriminate between the substrate and inhibitor. Lys 274 is essential for Fru-2,6-P_2 inhibition of FBPase (El-Maghrabi et al.,1992; Zhang and Fromm, unpublished observations).

Kinetic Studies in the Absence of Inhibitors--To gain some insight into the effects of the mutation on the kinetic mechanism and to confirm the finding that altering Arg 276 to methionine results in the total loss of Mg^{2+} cooperativity, we studied the kinetic behavior of both wild-type and mutant forms of FBPase in the absence of inhibitors. Figure 2A shows a double-reciprocal plot of 1/initial velocity against the concentration of 1/Fru-1,6-P_2. Figure 2B depicts a double-reciprocal plot of 1/initial velocity versus $1/[Mg^{2+}]^2$ with wild-type FBPase. From these two figures, it can be seen that, when Fru-1,6-P_2 concentration was varied at different fixed levels of Mg^{2+}, a family of lines intersecting in the second quadrant was obtained (Fig. 2A). The family of lines (Fig. 2B) for various $1/[Mg^{2+}]^2$ concentrations at different

fixed levels of Fru-1,6-P_2 was similar to those when Fru-1,6-P_2 was the varied substrate. The data shown in Fig. 2A and Fig. 2B gave much better fits to Equation 1 when n = 2 than when n = 1, e.g., the "Goodness of Fit" was 8% when n = 1 and 4% when n = 2. The form of Equation 1 is

$$\frac{1}{V}=\frac{1}{V_m}\left[1+\frac{K_a}{A^n}+\frac{K_b}{B}+\frac{K_{ia}K_b}{A^nB}\right] \quad (Eq.1)$$

where V, V_m, A, B, K_a, K_b, and K_{ia} represent the initial velocity, maximum velocity, the concentration of free Mg^{2+}, the concentration of free Fru-1,6-P_2, the Michaelis constant for Mg^{2+}, the Michaelis constant for Fru-1,6-P_2, and the dissociation constant for Mg^{2+}, respectively; n represents the Hill coefficient for Mg^{2+} with FBPase. When n = 1, there is no cooperativity; when n = 2, the binding of Mg^{2+} to FBPase is cooperative. Equation 1 is the fundamental rate equation based upon stochastic evaluation for the sequential kinetic mechanism shown in Scheme 1.



Scheme 1

$E + 2A = EA_2$, $K_{ia} = (E)(A)^2/EA_2$ (Eq. 2)

$E + B = EB$, $K_{ib} = (E)(B)/EB$ (Eq. 3)

$EA_2 + B = EA_2B$, $K_b = (EA_2)(B)/EA_2B$ (Eq. 4)

$EB + 2A = EA_2B$, $K_a = (EB)(A)^2/EA_2B$ (Eq. 5)

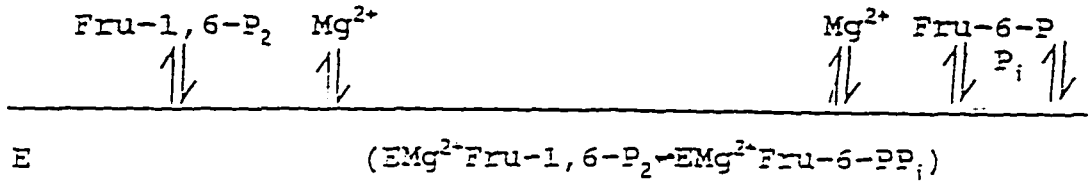
The data shown in Fig. 2A and Fig. 2B are consistent with previous reports that showed that Mg^{2+} activation and binding to FBPase exhibits cooperativity (Benkovic and deMaine, 1982; Nimmo and Tipton, 1975a,b; Tejwani, 1983). However, the kinetic data (shown in Fig. 3A and Fig. 3B) in the absence of inhibitors, with the mutant form of FBPase, gave excellent fits to Equation 1 when n = 1 and did not fit as well to Equation 1 when n = 2. In this case, the "Goodness of Fit" was 4% when n = 1 and 9% when n = 2. These results confirm the finding that altering Arg 276 to methionine results in the total loss of Mg^{2+} cooperativity with FBPase. Had Mg^{2+} ions bound cooperativity to FBPase, the data would have fitted to Equation 1 much better when n = 2 than when n = 1.

The mutation of Arg 276 to methionine, not only changes the Mg^{2+} cooperativity, but also changes the kinetic mechanism. From Fig. 3B, it can be seen that, when Mg^{2+} concentration was varied at different fixed concentrations of Fru-1,6-P_2, the family of lines intersected <u>on</u> the 1/velocity axis rather than in the second quadrant. This finding is important because the data shown in Fig. 3B represent a change in kinetic mechanism compared with the data shown in Fig. 2B; <u>i.e.</u>, the data in Fig. 2B represent either a Rapid-Equilibrium Random Bi Bi mechanism or a Steady-State Ordered Bi Bi mechanism, whereas the data shown in Fig. 3B represent a Rapid-Equilibrium Ordered Bi Bi mechanism (Fromm, 1975). For

the case of the mutant FBPase, Equation 1 can be written as

$$\frac{1}{V}=\frac{1}{V_m}\left[1+\frac{K_b}{B}+\frac{K_{ia}K_b}{AB}\right] \quad (Eq.6)$$

where all the terms in Equation 6 have the same meaning as those in Equation 1. Equation 6 is the initial rate equation for the Rapid-Equilibrium Ordered kinetic mechanism shown in Scheme 2.



Scheme 2

$E + A = EA, \quad K_{ia} = (E)(A)/(EA)$ (Eq. 7)

$EA + B = EAB, \quad K_b = (EA)(B)/(EAB)$ (Eq. 8)

$EAB \longrightarrow E + \text{products}$ (Eq. 9)

Discussion

The findings of this report demonstrate the loss of Mg^{2+} cooperativity and the change of kinetic mechanism when Arg 276 of porcine liver FBPase is mutated to methionine by site-directed mutagenesis. In addition, the mutation caused a significant decrease in enzyme activity; i.e., the k_{cat} of the mutant enzyme was only about 0.67% that of the wild-type enzyme.

Based on the crystal structure data (Zhang et al., 1993),

Based on the crystal structure data (Zhang et al., 1993), the ϵ NH_2 group of Arg 276 forms a salt bridge with the 1-phosphoryl oxygen O11 in the absence of metal ions. In the presence of metal ions, however, this salt bridge no longer exists because metal binding causes slight changes in orientation and position of the sugar ring and moves the 1-phosphoryl group towards the metal binding sites. The repositioning of the 1-phosphate is believed to be essential for activation and catalysis. Because Arg 276 does not bind Fru-1,6-P_2 in the presence of metal ions, mutation of Arg 276 to methionine should not affect substrate binding. Table 1 shows that the K_m of the mutant FBPase is similar to that of the wild-type enzyme. These results support the suggestion that no binding occurs between the substrate and Arg 276 in the presence of metal ions. On the other hand, Arg 276 must play important roles in the process of activation and catalysis. First, it may anchor the 1-phosphoryl group in the required position for metal binding, which is absolutely required for enzyme activity. Also, it may contribute to the orientation of various groups into proper positions for activation and catalysis. In addition, this residue may aid in the protonation of the ester oxygen O1, thus weakening the P-O bond so that nucleophilic attack by OH^- on the 1-phosphorus atom would be facilitated. These arguments are supported by the data shown in Table 1. It can be seen from Table 1 that porcine liver FBPase loses greater than 99% of

its activity when Arg 276 is mutated to methionine.

It has long been recognized that metal ions activate and bind cooperatively to FBPase (Benkovic and deMaine, 1982; Nimmo and Tipton, 1975a,b; Tejwani, 1983). A significant finding associated with this report is the complete loss of Mg^{2+} cooperativity with R276M mutant FBPase. The Hill coefficient of this mutant enzyme for Mg^{2+} is about 1. This conclusion is alluded to from the kinetic data evaluated by using either the ENZFITTER or MINITAB programs. These results indicate that replacing Arg 276 of FBPase by methionine essentially causes the complete loss of Mg^{2+} cooperativity, but the rationale behind the metal cooperativity and its loss is unclear. Nimmo and Tipton (1975a,b) showed that the plots of velocity against Mg^{2+} are sigmoidal at neutral pH but that they are hyperbolic at pH 9.6. Site-directed mutagenesis at the metal binding sites (Chen et al.,1993; El-Maghrabi et al.,1993) did not significantly alter the Mg^{2+} cooperativity. However, Mg^{2+} cooperativity was totally lost when Gly 122 was mutated to alanine (Zhang et al.,1995). It is believed that the mutation can reduce the flexibility of the H4 (residue 123-127) region or lock the enzyme in a conformation that blocks signal transmission and suppresses the cooperativity for Mg^{2+}. All these findings suggest, but do not provide conclusive proof, that divalent metal ion cooperativity is inter- rather than intra-subunit. The X-ray diffraction studies of FBPase by the Lipscomb group (Zhang et al., 1994;

Ke et al., 1991) have provided a basis for AMP cooperativity. The enzyme forms a dimmer of dimmers in which the R and T states are in equilibrium. When AMP binds, the T state is induced, and the substrate binds to the R state. This model provides a rational explanation for a Hill coefficient of 2 for AMP. The results of the present study may lead to a better understanding of the cooperativity phenomenon for divalent ion binding to FBPase at the molecular level when the three-dimensional structure of the R276M mutant FBPase becomes available.

It is well documented that AMP and Fru-2,6-P_2 are potent synergistic inhibitors of FBPase (Chen et al.,1994; Van Schaftingen et al., 1980a,b). The binding of AMP to wild-type FBPase exhibits cooperativity (Taketa and Pogell, 1965; Nimmo and Tipton, 1975a; Liu and Fromm, 1990; Chen et al., 1994). Site-directed mutagenesis at the AMP binding site leads to a total loss of AMP cooperativity with FBPase (Chen et al., 1994). The kinetic data of AMP inhibition with R276M mutant FBPase gave excellent fits to a cooperative model. Also, the K_i of R276M enzyme for AMP increased only 2-fold relative to that of wild-type enzyme (shown in Table 1). These results suggest that Arg276 may not be involved in AMP inhibition and cooperativity. The other regulator, Fru-2,6-P_2, is believed to have two functions in regulating FBPase: one is that it is a competitive inhibitor of Fru-1,6-P_2 and competes with the substrate for the enzyme's active site (Van Schaftingen and

Hers, 1980; Pilkis et al.,1981; Ganson and Fromm, 1982; Ke et al., 1989, 1990); the other function is to enhance the effect of AMP by making AMP "stickier" to the enzyme (Liu and Fromm, 1988). Altering Arg 276 to methionine did not cause a significant increase in K_i for Fru-2,6-P_2 (Table 1). The results suggest that Arg 276 may not be directly involved in Fru-2,6-P_2 inhibition.

The kinetics of both the forward and reverse reactions of FBPase have been studied in detail with enzymes from a number of sources (Nimmo and Tipton, 1975a; Marcus et al., 1973; Dudman et al., 1978; Caperelli et al., 1978; Casazza et al., 1979; Stone and Fromm, 1980; Ganson and Fromm, 1982). Liu and Fromm (1990) studied the kinetic mechanism at alkaline pH (9.6) and found the kinetic mechanism to be Rapid-Equilibrium Random Bi Bi. Kinetic results of this study have shown that double-reciprocal plots involving magnesium ion are intersecting (Fig. 2B and Fig. 3B), indicating a sequential mechanism for Mg^{2+} and Fru-1,6-P_2. However, the plots of 1/velocity against $1/[Mg^{2+}]^2$ in the case of wild-type FBPase intersect in the second quadrant (Fig. 2B), whereas the plots of 1/velocity versus $1/[Mg^{2+}]$ in the case of R276M mutant FBPase intersect on the 1/velocity axis (Fig. 3B). The data presented in Fig. 2B are consistent with either a Rapid-Equilibrium Random or a Steady-State Ordered Bi Bi mechanism. These results are in harmony with previous reports (Zhang et al., 1995; Liu and Fromm, 1990; Marcus et al., 1973). On the

other hand, the data shown in Fig. 3B support a Rapid-Equilibrium Ordered Bi Bi mechanism as shown in Scheme II. From Scheme II and Equation 6, it can be seen that Fru-1,6-P_2 binds to the enzyme before Mg^{2+} adds. Another example of switching kinetic mechanisms from Rapid-Equilibrium Random Bi Bi to Ordered Bi Bi was reported by Schimerlik and Cleland (1973) who found that lowering the pH from 8 to 7 changed the kinetic mechanism for creatine kinase.

Benkovic et al. (1978) suggested that catalysis requires the sequential addition of metal and substrate in the order: structural metal, substrate, and catalytic metal to form a catalytically competent quaternary complex of enzyme-M_1-M_2-Fru-1,6-P_2 with respect to Mn^{2+} or Zn^{2+} binding. On the other hand, no direct evidence from binding studies support the existence of structural and catalytic sites for Mg^{2+}. Also, crystallographic data (Zhang et al. (1993) suggest that two metal ions with respect to Mn^{2+} or Zn^{2+} are associated with each enzyme subunit; however, only one Mg^{2+} per subunit was found to bind FBPase. However, the structural and catalytic sites for Mg^{2+} can be inferred from kinetic studies (Benkovic and deMaine, 1982). On the basis of the experimental data of this report, we postulate that one of the roles of Arg 276 might be to direct and ensure the binding of the structural metal. The enzyme loses its ability to bind the structural metal when Arg 276 is replaced by methionine, and the catalytic metal can only bind to the enzyme after the binding

of Fru-1,6-P_2. If this is true, it can explain, not only the change of kinetic mechanism, but also the loss of metal cooperativity and the significant decrease of the enzyme activity; i.e., without the structural metal, the catalytic metal cannot efficiently direct the 1-phosphoryl group to the orientation required for catalysis.

References

Benkovic, P.A., Caperelli, C.A., deMaine, M.M., and Benkovic, S.J.(1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 2185-2189.

Benkovic, S.J., and deMaine, M.M. (1982) Adv. Enzymol. Relat. Areas Mol. Biol. **53**, 45-82.

Burton, V.A., Chen, M., Ong, E.E., Ling, T., Fromm, H.J., and Stayton, M.M. (1993) Biochem. Biophys. Res. Commun. **192**, 511-517.

Caperelli, C.A., Frey, W.A., and Benkovic, S.J. (1978) J. Biol. Chem. **253**, 5952-5956.

Casazza, J.P., Stone, S.R., and Fromm, H.J. (1979) J. Biol. Chem. **254**, 4661-4665.

Chen, L., Remesh, H., Chen, M., and Fromm, H.J. (1993) Arch. Biochem. Biophys. **307**, 350-354.

Chen, M., Chen, L., and Fromm, H.J. (1994) J. Biol. Chem. **269**, 5554-5558.

Dudman, N.P.B., deMaine, M.M., and Benkovic, S.J. (1978) J. Biol. Chem. **253**, 5712-5718.

El-Maghrabi, M.R., Austin, L.R., Correia, J.J., and Pilkis,

S.J. (1992) J. Biol. Chem. **267**, 6526-6530.

El-Maghrabi, M.R., Gidh-Jain, M.,Austin, L.R., and Pilkis, S.J. (1993) J. Biol. Chem. **268**, 9466-9472.

Fromm, H.J. (1975) Initial Rate Enzyme Kinetics, pp.33-37, Springer-Verlag, West Berlin.

Ganson, N.J., and Fromm, H.J. (1982) Biochem. Biophys. Res. Commun. **108**, 233-239.

Gomori, G. (1943) J. Biol. Chem. **148**, 139-149.

Hers, H.G., and Hue, L.(1983) Annu. Rev. Biochem. **52**, 617-653.

Hers, H.G., and Van Schaftingen, E. (1982) Biochem. J. **206**, 1-12.

Ke, H., Liang, J.-Y., Zhang, Y., and Lipscomb, W.N. (1991) Biochemistry **30**, 4412-4420.

Ke, H., Thorpe, C.M., Seaton, B.A., Marcus, F., and Lipscomb, W.N. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 1475-1479.

Ke, H., Thorpe, C.M., Seaton, B.A., Lipscomb, W.N.,and Marcus, F. (1990) J. Mol. Biol. **212**, 513-539.

Krebs, H.A. (1963) in Advances in Enzyme Regulation (Weber, G., ed) Vol.1, pp.385-400, Pergamon Press Ltd., London.

Leatherbarrow, R.J. (1987) Enzfitter: A Nonlinear Regression Data Analysis Program for the IBMPC, pp.13-75, Elsevier Science Publishers BV, Cambridge, United Kingdom.

Liu, F., and Fromm, H.J. (1988) J. Biol. Chem. **263**, 9122-9128.

Liu, F., and Fromm, H.J. (1989) Biochem. Biophys. Res. Commun. **161**, 689-695.

Liu, F., and Fromm, H.J. (1990) J. Biol. Chem. **265**, 7401-7406.

Marcus, C.J., Celler, A.M., and Byrne, W.L. (1973) J. Biol. Chem. **248**, 8567-8573.

Marcus, F. (1981) in The Regulation of Carbohydrate Formation and Utilization in Mammals (Veneziale, C.M., ed) pp.269-290, University Park Press, Baltimore.

Marcus, F., Edelstein, L., Reardon, I., and Heinrikson, R.L. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 7161-7165.

McGrane, M.M., El-Maghrabi, M.R., and Pilkis, S.J. (1982) J. Biol. Chem. **258**, 10445-10454.

Nakamaye, K., and Eckstein, F. (1986) Nucleic Acids Res. **14**, 9679-9898.

Nimmo, H.G., and Tipton, K.F. (1975a) Eur. J. Biochem. **58**, 567-574.

Nimmo, H.G., and Tipton, K.F. (1975b) Eur. J. Biochem. **58**, 575-585.

Pilkis, S.J., El-Maghrabi, M.R., Pilkis, J., and Claus, T.H. (1981) J. Biol. Chem. **256**, 3619-3622.

Pilkis, S.J., El-Maghrabi, M.R., and Claus, T.H. (1988) Annu. Rev. Biochem. **57**, 755-783.

Pontremoli, S., and Traniello, S. (1975) Methods Enzymol. **42**, 369-374.

Pontremoli, S., and Traniello, E., Michetti, M., Salamino, F., Sparatore, B., and Horecker, B.L. (1982) Arch. Biochem. Biophys. **218**, 609-613.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463-5467.

Scheffer, J.E., and Fromm, H.J. (1986) Biochemistry **25**, 6659-6665.

Schimerlik, M.I., and Cleland, W.W. (1973) J. Biol. Chem. **248**, 8418-8423.

Stone, S.R., and Fromm, H.J. (1980) Biochemistry **19**, 620-625.

Taketa, K., and Pogell, B.M. (1965) J. Biol. Chem. **240**, 651-662.

Tejwani, G. (1983) Adv. Enzymol. Relat. Areas Mol. Biol. **54**, 121-193.

Van Schaftingen, E., and Hers, H.G. (1980) Biochem. Biophys. Res. Commun. **96**, 1524-1531.

Van Schaftingen, E., Hue, L., and Hers, H.G. (1980a) Biochem. J. **192**, 887-895.

Van Schaftingen, E., Hue, L., and Hers, H.G. (1980b) Biochem. J. **192**, 897-901.

Zhang, R., Chen, L., Villeret, V., and Fromm, H.J. (1995) J. Biol. Chem. **270**, 54-58.

Zhang, Y., Liang, J.-Y., Huang, S., Ke, H., and Lipscomb, W.N. (1993) Biochemistry **32**,1844-1857.

Zhang, Y., Liang, J.-Y., Huang, S., and Lipscomb, W.N. (1994) J. Mol. Biol. **244**, 609-624.

CHAPTER 4. KINETICS AND MECHANISMS OF ACTIVATION AND INHIBITION OFPORCINE LIVER FRUCTOSE-1,6-BISPHOSPHATASE BY MONOVALENT CATIONS[1]

A paper published in Biochemistry[2]

Rulin Zhang[3,4], Vincent Villeret[5], William N. Lipscomb[5], and Herbert J. Fromm[3,6]

Abstract

K^+ and Li^+ were used to study the kinetic effects of monovalent cations on porcine liver fructose-1,6-bisphosphatase (FBPase). At saturating fructose 1,6-bisphosphate (FBP) concentrations, Li^+ was found to be a linear noncompetitive inhibitor with respect to Mg^{2+}. K^+ was found to activate the wild-type enzyme at low concentrations (K_m = 17 mM) and to inhibit the enzyme at high concentrations (K_{IK+} = 68 mM). A steady-state random ter mechanism was proposed, and a mathematical equation was derived to account

---

[1]This research was supported in part by Research Grants NS 10546 and GM06920 from the National Institutes of Health, United State Public Health Service, and Grant MCB-9218763 from the National Science Foundation. This is Journal Paper J-16406 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Project 3191.
[2]Reprinted with permission of Biochemistry, 1996, 35, 3038-3043.
[3]Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011.
[4]Primary researcher and author under the supervision of Herbert J. Fromm.
[5]Gibbs Chemical Laboratory, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138.
[6]To whom all correspondence should be addressed.

for the Mg^{2+} and K^+ kinetics and activation of FBPase. Interestingly, when Glu 280 was mutated to glutamine by site-directed mutagenesis, K^+ lost the ability to activate the enzyme and became a noncompetitive inhibitor with respect to Mg^{2+}. These kinetic data suggest that K^+ has two distinct sites: One is a high-affinity activation site, and the other a low-affinity inhibition site. Glu 280 is essential for allowing K^+ to bind at the activation site. Due to the geometric constraints and its small atomic radius, Li^+ can bind only at the inhibitory site. It is postulated that monovalent cations activate FBPase by helping the Arg 276 residue "deshield" the partial negative charge on the 1-phosphoryl group of the substrate so that nucleophilic attack on the 1-phosphorus atom is facilitated. In addition, monovalent cations may, along with Mg^{2+} ions and surrounding residues of the protein, help orient the 1-phosphoryl group so as to achieve the optimal position required for catalysis. Monovalent cations inhibit FBPase by either distorting the geometry of the active site or by retarding turnover or product release.

Introduction

Fructose-1,6-bisphosphatase (FBPase, EC3.1.3.11) plays an important role in the regulation of gluconeogenesis (Krebs, 1963; Marcus, 1981; Hers & Hue, 1983; Pilkis et al., 1988). In the presence of a divalent metal ion, it catalyzes the

hydrolysis of fructose 1,6-bisphosphate (FBP) to form fructose 6-phosphate (F6P) and inorganic phosphate. The reaction is competitively inhibited by fructose 2,6-bisphosphate (Fru-2,6-P_2) and noncompetitively inhibited by AMP (Liu & Fromm, 1990). These two molecules act synergistically to inhibit FBPase (Hers & Van Schaftingen, 1982). The role of AMP is thought to prevent divalent metal binding to FBPase, and the divalent metal binding is required for FBPase activity (Gomori, 1943; Benkovic & deMaine, 1982). Fru-2,6-P_2 is believed to have two functions in regulating FBPase: One is that it is a potent competitive inhibitor of FBP and competes with the substrate for the enzyme's active site (Ke et al., 1989; Van Schaftingen & Hers, 1980; Ke et al., 1990); the other is to keep AMP on the enzyme (Liu & Fromm, 1988), thus enhancing the action of AMP.

It is known that FBPase is a homotetramer with a subunit molecular mass of 37 KDa (Marcus et al., 1982; Burton et al., 1993). It has been shown from binding studies (Benkovic et al., 1978) and the crystallographic structure (Zhang et al., 1993) that there are two divalent metal-binding sites per subunit: a high-affinity "structural" site (M1) and a low-affinity "catalytic" site (M2) (Benkovic et al., 1978) and that catalysis requires the formation of a catalytically competent quaternary complex of enzyme-M1-M2-FBP; however, in the case of Mg^{2+} ions, only one ion binds per subunit based on kinetic (Liu & Fromm, 1990) and x-ray diffraction studies

(Zhang et al., 1993), and there is no evidence that the M2 site is occupied by Mg^{2+} (Zhang et al., 1993). A monovalent cation is needed for the enzyme to achieve its maximal activity (Hubert et al., 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Marcus & Hosey, 1980). Among the monovalent cations studied, K^+, NH_4^+, and Tl^+ are the best activators, whereas Li^+ is a strong inhibitor; however, how these monovalent cations affect the activity of FBPase remains unclear. Tejweni et al. (1976) proposed that the activation effect of K^+ was to overcome inhibition of the enzyme by high concentrations of divalent metal ions. Xu et al. (1993) suggested that activation by monovalent cations might result from the binding of K^+ at a site distinct from the catalytic site. Marcus (1975) reported that activation of FBPase by K^+ could be abolished by modifying arginyl residues with 2,3-butanedione in the presence of AMP. He also found that only one arginyl residue per subunit played an essential role in monovalent cation activation of the enzyme. This arginyl residue was thought to be in the substrate binding site on the basis of the fact that no loss of monovalent cation activation occurred when modification was carried out in the presence of AMP plus the substrate. Very probably, this residue is Arg 276, which forms a salt-bridge in the absence of divalent cations (Zhang et al., 1993). This arginyl residue plays important roles in both enzyme activity and Mg^{2+} cooperativity, and in determining the kinetic mechanism of

FBPase (Zhang & Fromm, 1995).

In order to gain insight into the effects and mechanism of monovalent cations on FBPase and the location of the binding site, we have studied the kinetic effects of monovalent cations on FBPase by using K^+ and Li^+ as models of activation and inhibition, respectively. Here, we report that Li^+ is a noncompetitive inhibitor with respect to Mg^{2+}, whereas K^+ activates wild-type FBPase at low concentrations but inhibits the enzyme at high levels. Our results, together with crystallographic studies of FBPase in the presence of monovalent cations (Villeret et al., 1995), identify the different binding sites of K^+ and Li^+. From these studies, the molecular mechanism of action for these monovalent cations are discussed. This is thought to be the first attempt to explain the role of monovalent cations on FBPase at the molecular level based on the structure and function of the enzyme.

Experimental Procedures

Materials--NADP, fructose 1,6-bisphosphate, fructose 2,6-bisphosphate, AMP, Hepes, and Tris were purchased from Sigma (St. Louis, MO). Glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim (Indianapolis, IN). Distilled deionized water was used in all experiments. All other reagents were of the highest purity available commercially. A mutant of recombinant porcine liver

FBPase, Glu280→Gln, was obtained by site-directed mutagenesis as described elsewhere (Chen et al., 1993). Recombinant and mutant forms of porcine liver FBPase were prepared and purified as previously described (Chen et al., 1993) with slight modifications. The pET-11a expression vector carrying either the wild-type or mutant FBPase gene was transformed into E. coli DE657 host cell, a strain deficient in the FBPase gene. Mutant forms of the enzyme were obtained in yields comparable to those of the wild-type enzyme. Porcine liver and kidney FBPase are identical in their primary sequences (Burton et al., 1993).

Protein Assay--Total protein was measured by the Bio-Rad method with bovine serum albumin (from Sigma) as a standard.

Circular Dichroism Spectrometry--CD studies on the wild-type and mutant form of FBPase were carried out in 50 mM Tris-HCl buffer (pH7.5) at room temperature in an AVIV CD spectrometer model 62DS kindly supplied by Dr. Earl Stellwagen at the University of Iowa. Samples were placed in a 1-mm cuvette, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was calibrated to remove the background of the buffer and smoothed by using a program in the computer of the spectrometer.

Kinetic Studies--FBPase activity during purification and the specific activity of pure enzyme were measured by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (Pontremoli & Traniello,

1975). All other kinetic experiments were done using a fluorometric assay (Liu & Fromm, 1990) at pH 7.5 (50 mM Hepes buffer) and 24°C. The initial-rate data were analyzed for kinetic mechanisms by using a MINITAB language program with an α value of 2.0 (Liu & Fromm, 1990).

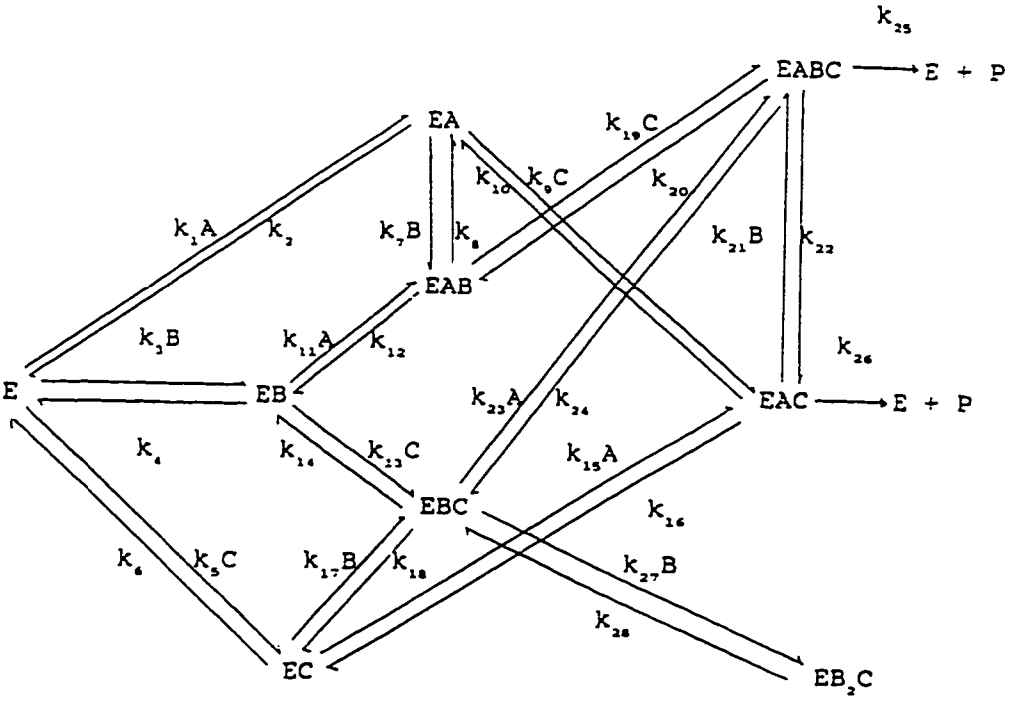
Results

Enzyme Quality--The purity of wild-type recombinant FBPase and the Glu280Gln mutant of porcine FBPase were evaluated by SDS-PAGE. The proteins were greater than 95% pure by using the criterion of electrophoresis. Also, no discernible degradation of the proteins was observed (data not shown).

Secondary Structure Analysis--The secondary structures of recombinant wild-type and the Glu280Gln mutant of FBPase were analyzed by CD spectrometry. The purpose of this study was to determine whether localized or global structural alterations were induced in the mutant. The CD spectral data showed that the spectrum of the mutant was essentially superimposable on that of the wild-type enzyme (data not shown). These results suggest that no major conformational changes occurred in FBPase when Glu 280 was mutated to glutamine by using CD as a criterion of secondary protein structure.

Effects of $K^{\pm}$ on Wild-type FBPase--K^{+} plays a dual role in affecting the activity of wild-type FBPase. It can activate and inhibit the enzyme in a concentration-dependent manner.

Fig. 1 illustrates the effects of K^+ on the kinetics of Mg^{2+} activation and inhibition of FBPase at saturating levels of FBP. From Fig. 1 it can be seen that, when 1/v vs. $1/[Mg^{2+}]^2$ is plotted at different fixed concentrations of K^+, the lines are linear and the intercepts of the lines on 1/v axis decrease while the slopes increase as the concentrations of K^+ increases. A stochastic approach was used to fit the data shown in Fig. 1 and then to explain the results shown in Fig. 1. The simplest and most feasible rationale for the data in Fig. 1 is described by the mechanism depicted in Scheme 1, which is a steady-state random ter kinetic model as shown in Scheme 1,



Scheme 1

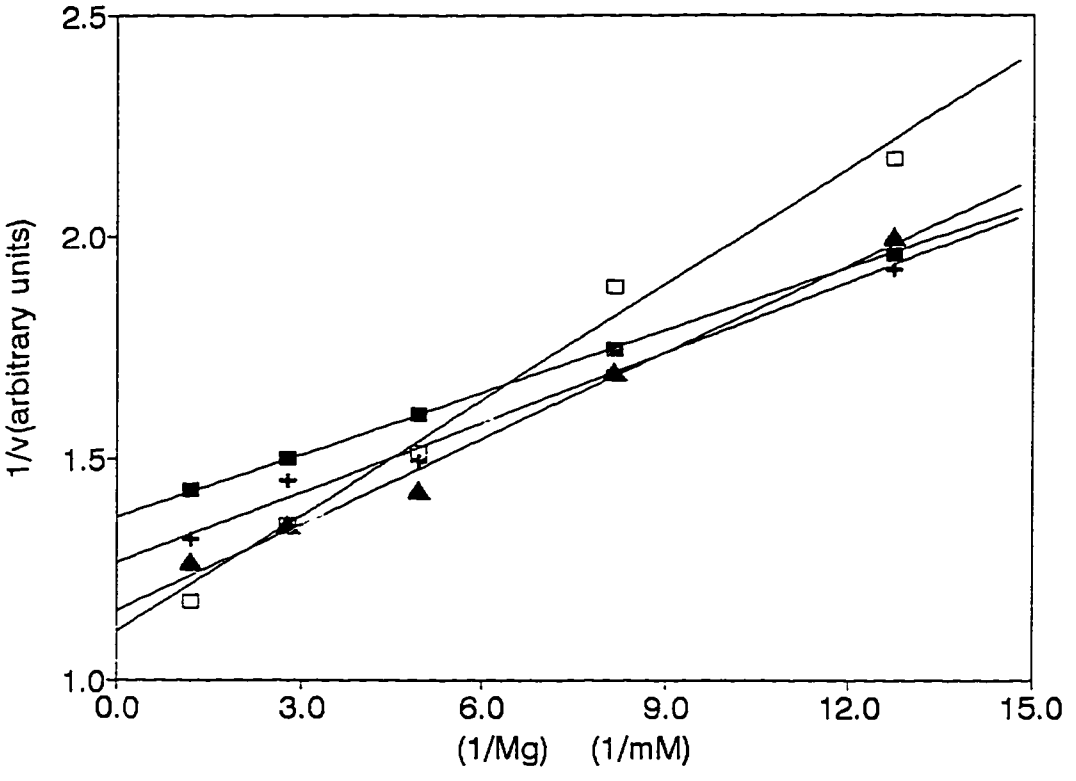


Fig. 1 Plot of reciprocal of initial velocity in arbitrary fluorescent units against reciprocal of $[Mg^{2+}]^2$ at different concentrations of K^+ with wild-type FBPase. The concentrations of K^+ are 25 mM (■), 30 mM (+), 35 mM (▲), 45 mM (□). These concentrations include the amount of K^+ used to adjust the pH of Hepes buffer. The lines are theoretical and based on eq 2. All the points are experimentally determined.

where A = Mg^{2+}, B = K^+, and C = FBP. Scheme 1 depicts a paradigm of K^+ and Mg^{2+} activation of FBPase based upon a number of well-documented investigations reported in the literature. These include the observations that there is an absolute requirement for divalent metal ions by FBPase for activity (Gomori, 1943; Benkovic & deMaine, 1982), that monovalent cations such as K^+ can onlyserve to activate FBPase in the presence of divalent cations (i.e., in the presence of K^+, but in the absence of divalent ions such as Mg^{2+}, FBPase is inactive), that only one Mg^{2+} ion binds per FBPase subunit (Zhang et al., 1993), that the kinetic mechanism of FBPase at pH 9.5 is rapid-equilibrium random Bi Bi (Liu & Fromm, 1990), and that, at saturating Mg^{2+}, the FBPase reaction in the nonphysiological direction is steady-state random Bi Bi (Stone & Fromm, 1980). The model described in Scheme 1 was thus proposed in the context of the previously reported kinetic studies on the kinetic mechanism and activation of FBPase by Mg^{2+} and K^+ ions. The initial-rate equation for this kinetic model is

$$v=\frac{[k_{25}(EABC)+k_{26}(EAC)]E_0}{E+EA+EB+EC+EAB+EAC+EBC+EB_2C+EABC} \quad (1)$$

The determinants for E, EA, EB, EC, EAB, EAC, EBC, EB_2C and EABC, were obtained by using a computer program (Fromm, 1975). When these determinants were substituted into eq 1, a very complex equation containing 957 terms in the numerator and 6116 terms in the denominator of eq 1 was obtained (not

shown). Since the kinetic studies were performed at a saturating FBP concentration (12 μM), most of the terms in numerator and denominator were eliminated. To simplify the equation further, three assumptions were made: a) all forward direction steps are much faster than the reverse direction steps; b) Mg^{2+} adds more rapidly to the free enzyme and the enzyme-substrate complexes than does K^+; c) K^+ binds to the enzyme-Mg^{2+}-FBP complex much faster than either the breakdown of the competent quaternary complex of enzyme-Mg^{2+}-FBP-K^+ to products or the dissociation of Mg^{2+} from this complex. These assumptions are based on the kinetics of the system, data from the literature (Gomori, 1943; Benkovic & deMaine, 1982; Hubert et al., 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Marcus and Hosey, 1980), and the crystal structure of FBPase (Zhang et al., 1993) which shows that, in the absence of divalent metal ions, the ε-amino group of Arg276 forms a salt-bridge with the 1-phosphoryl group of FBP. However, this salt-bridge no longer exists in the presence of divalent ions because the bound divalent ions displace the 1-phosphoryl group toward the metal-binding site. Thus, forming a salt-bridge between Arg276 and FBP may hinder K^+ binding to the free enzyme, but, the binding of Mg^{2+} to the enzyme disrupts this salt-bridge, which provides room for K^+, so that K^+ binding is facilitated. On the basis of these assumptions, the complex equation was simplified to eq 2:

$$\frac{k_{25}E_o}{v} = 1 + \frac{(k22/k21)}{(k26/k21)+B} + \frac{(k_{17}k_{25})}{(k_{15}k_{23})}\left[\frac{(k_{18}/k_{17})+B}{A^2}\right](1+\frac{B}{K_{IB}}) \quad (2)$$

in which $k_{21} >> k_{26}$ and $k_{17} >> k_{16}$ based on the above assumptions. Thus, eq 2 is further simplified to eq 3:

$$\frac{1}{v} = \frac{1}{V_m}\left[1+\frac{K_a B}{A^2}(1+\frac{B}{k_{IB}})+\frac{K_b}{B}\right] \quad (3)$$

where v, A, B represent initial velocity, concentration of Mg^{2+}, and concentration of K^+, respectively; $V_m = k_{25}E_0$, which is the maximum velocity; $K_a = (k_{17}k_{25})/(k_{15}k_{23})$; K_b is the activation constant for K^+, and K_{IB} is the inhibition constant for K^+. The data in Fig. 1 fit very well to eq 3; e.g., the "Goodness of Fit" was 1.5%. The kinetic constants for the effects of K^+ on wild-type FBPase are summarized in Table I. From Table I it can be seen that K_{IB} is about 4-fold higher than K_b.

Table I. Kinetic constants for the effects of K^+ on wild-type FBPase

Constant	Enzyme interaction	Value
K_b	FBP-E-Mg^{2+} + K^+ = K^+-E-Mg^{2+}	17 ± 0.23 mM
K_{IB}	K^+-E-FBP + K^+ = K_2^+-E-FBP	68 ± 2.1 mM

The data in Fig. 1 are not consistent with a Rapid-Equilibrium Random model in which all the steps in Scheme 1 are in rapid equilibrium relative to the breakdown of the EAC and EABC complexes to products. In addition, the data do not fit to the model where B binds the EABC complex.

Effects of K^+ on Glu280Gln Mutant FBPase--

Crystallographic studies have shown (Fig. 4) that metal site 1 is defined by Glu280, Glu97, Asp118, Asp121, and the 1-phosphoryl group of FBP (Zhang et al., 1993); metal site 3, which is thought to be the putative K^+ activation site, is defined by Glu280, Arg276, and the 1-phosphoryl group of FBP (Villeret et al., 1995). Therefore, Glu280 is thought to be important for metal ion action. A mutant, Glu280Gln, was prepared and characterized, the kinetic effects of K^+ on this mutant form of FBPase were also studied. The mutation caused dramatic decrease in enzyme activity, the k_{cat} of the mutant enzyme is 0.015 s^{-1}, which is 0.1% of that of wild-type enzyme. The kinetic data are shown in Fig. 2. From Fig. 2, it can be seen that when 1/v vs. $1/[Mg^{2+}]$ is plotted at different fixed concentrations of K^+, a noncompetitive inhibition pattern was obtained. These findings suggest that K^+ is a noncompetitive inhibitor with respect to Mg^{2+} when Glu280 of FBPase is changed to glutamine. The data in Fig. 2 fit well to eq 4 (the "Goodness of Fit" was 5.3%) but did not fit to other models such as a competitive inhibition model, a rapid-equilibrium random Bi Bi model, or the steady-state

random model shown in Scheme 1. The form of eq 4 is

$$\frac{1}{v}=\frac{1}{V_m}\left[1+\frac{I}{K_{ii}}+\frac{K_a}{A}+\frac{K_aI}{K_iA}\right] \quad (4)$$

where v, V_m, A, I, K_i, and K_{ii}, represent the initial velocity, maximum velocity, the concentration of Mg^{2+},

Table II. Kinetic constants for the effects of K^+ on Glu280Gln FBPase

Constant	Enzyme interaction	Value (mM)
K_a	$E + Mg^{2+} = E\text{-}Mg^{2+}$	1.4 ± 0.14
K_i	$E + K^+ = K^+\text{-}E$	102 ± 56.0
K_{ii}	$E\text{-}Mg^{2+} + K^+ = K^+\text{-}E\text{-}Mg^{2+}$	222 ± 43.0
K_{iii}	$K^+\text{-}E + Mg^{2+} = K^+\text{-}E\text{-}Mg^{2+}$	3.4 ± 0.51

Table II. Kinetic constants of the effects of Li^+ on wild-type FBPase

Constant	Enzyme interaction	Value
K_a	$E + Mg^{2+} = E\text{-}Mg^{2+}$	0.50 ± 0.04 mM^2
K_i	$E + Li^+ = Li^+\text{-}E$	0.20 ± 0.05 mM
K_{ii}	$E\text{-}Mg^{2+} + Li^+ = Li^+\text{-}E\text{-}Mg^{2+}$	1.3 ± 0.57 mM
K_{iii}	$Li^+\text{-}E + Mg^{2+} = Li^+\text{-}E\text{-}Mg^{2+}$	3.3 ± 0.05 mM

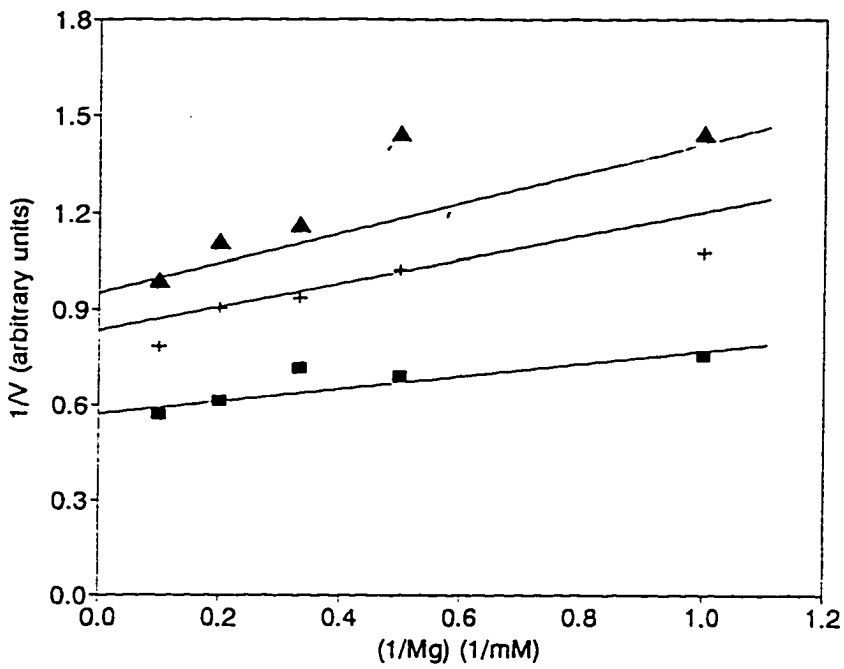


Fig. 2 Plot of reciprocal of initial velocity in arbitrary fluorescent units versus reciprocal of $[Mg^{2+}]$ at different concentrations of K^+ with Glu280Gln mutant FBPase. The concentrations of K^+ are 25 mM (■), 125 mM (+), 175 mM (▲). These concentrations include the amount of K^+ used to adjust the pH of Hepes buffer. The lines are theoretical and based on eq 3, and the points are experimentally determined.

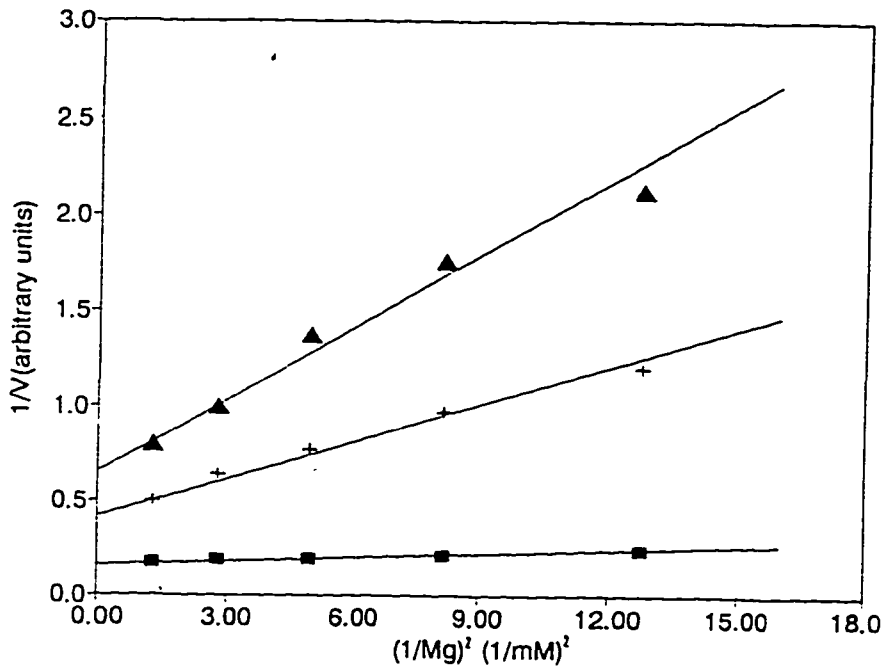


Fig. 3 Plot of reciprocal of initial velocity in arbitrary fluorescent units versus reciprocal of $[Mg^{2+}]^2$ at different concentrations of Li^+ with wild-type FBPase. The concentrations of Li^+ are 0 mM (■), 2 mM (+), and 4 mM (▲). The lines are theoretical and based on eq 3, where the A terms are second order and the points are experimentally determined. The assay solutions contained 25 mM K+ which was used to adjust the pH of Hepes buffer. When 50 mM Tris-HCl buffer (pH 7.5) was used (no K^+), identical results were obtained.

the concentration of K^+, and the inhibition constants for K^+, respectively. It is noteworthy that the A term in eq 4 is first power instead of second power. These results suggest that the mutation caused FBPase to lose Mg^{2+} sigmoidicity. When the concentration of Hepes-Na^+ buffer (pH7.5) was varied from 30 mM to 250 mM, enzyme activity was not affected. This result indicates that the effect of K^+ on Glu280Gln FBPase is not due to ion strength. Eq 4 is the rate equation derived from a conventional noncompetitive inhibition model. The kinetic constants for the effect of K^+ on Glu280Gln mutant FBPase are shown in Table II.

Effects of Li^+ on Wild-type FBPase--Fig. 3 depicts the kinetic effects of Li^+ on wild-type FBPase. At saturating FBP concentration (12 μM), a noncompetitive inhibition pattern was obtained when 1/v was plotted versus $1/[Mg^{2+}]^2$. The data in Fig. 3 gave excellent fits to eq 4 where the A term was raised to the second power (Mg^{2+} sigmoidicity was not affected by Li^+ ion). The "Goodness of Fit" was 3.0%. When the data in Fig. 3 were fit to other models such as a competitive inhibition model, a rapid-equilibrium random Bi Bi model, or the steady-state random model as shown in Scheme 1, the "Goodness of Fit" value was at least 20%. The kinetic constants for the effects of Li^+ on FBPase are summarized in Table III. From Tables I and III, it can be seen that the affinity of FBPase for Li^+ is much higher than for K^+.

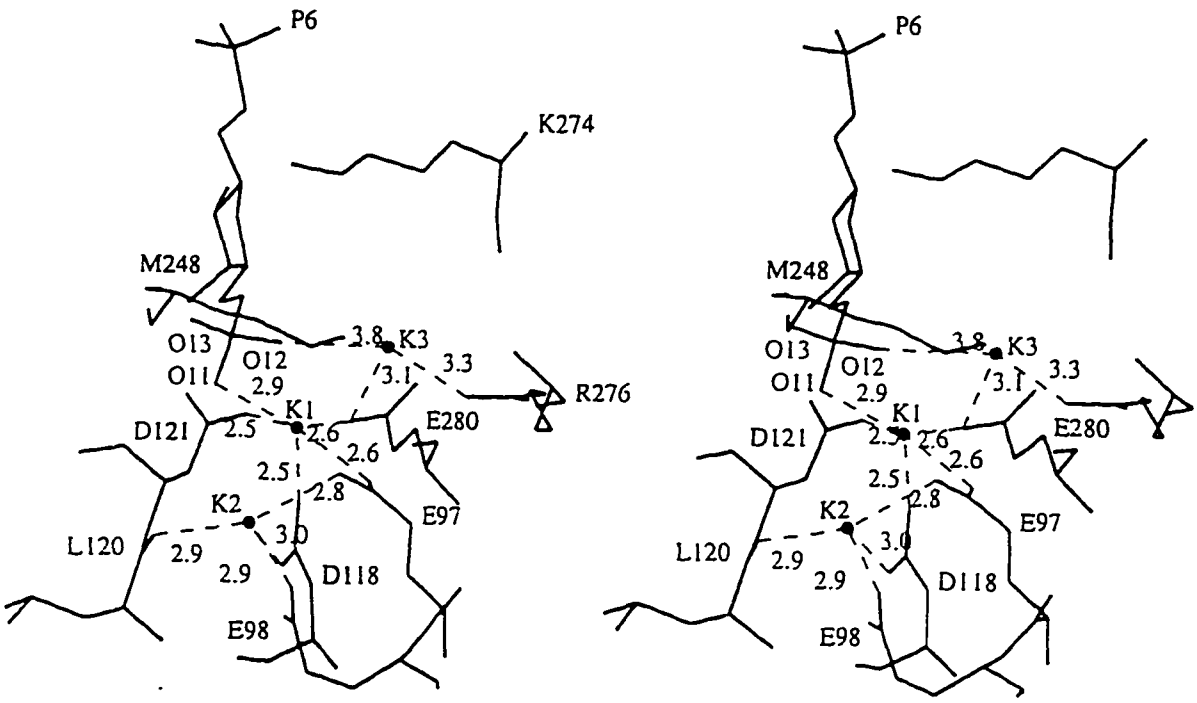


Fig. 4 Stereo view of the metal binding region in the presence of the substrate analogue anhydro-D-glucitol-1,6-bisphosphate (AhG-1,6-P_2) and potassium ions, as observed in the crystal structure (Villeret et al., 1995). P6 refers to the 6-phosphoryl group of AhG-1,6-P_2; O11, O12, and O13 are 1-phosphoryl oxygens. K1, K2, and K3 refer to potassium ions at sites 1, 2, and 3, respectively. Distances between K^+ ions and their ligands are indicated. K1 and K2 are bound at the divalent metal sites, and K3 at the specific site for K^+ or Tl^+ ions. K1 and K2 are 3.9 Å apart; K1 and K3 are 3.4 Å apart.

Discussion

The results of this report are consistent with the chemical modification studies reported by Marcus (Marcus, 1975) and the crystallographic studies from Lipscomb's laboratory (Villeret et al., 1995). Marcus found that modification of pig kidney FBPase with 2,3-butanedione in the presence of AMP resulted in the loss of activation of the enzyme by monovalent cations, but no loss of monovalent cation activation was observed when the modification was carried out in the presence of both AMP and FBP. In addition, Marcus found that one arginyl residue per subunit plays an essential role in monovalent cation activation of the enzyme. It is now clear that this residue is probably Arg276 because this arginyl residue alone, together with Glu280 and the 1-phosphoryl group of FBP, define the K^+ and Tl^+ binding site (Villeret et al., 1995). Fig. 4 illustrates the binding of K^+ at the divalent metal-binding sites (K1 and K2), and at the monovalent cation activator site (K3). The third metal-binding site reported for FBPase is specific for monovalent cations. Metal sites 1 and 2 are divalent cation-binding sites (Zhang et al., 1993). Li^+ can not be seen by X-ray analysis, but kinetic evidence in this report and indirect evidence from the crystal structure data (Villeret et al., 1995) have shown that it binds at metal site 1, which is defined by Glu280, Glu97, Asp118, Asp121, and the 1-phosphoryl group of FBP (Zhang et al., 1993; Villeret et al., 1995). The

kinetic data presented in this report suggest that K^+ can bind to two sites: one activation site with high affinity and one inhibition site with low affinity. It is probable that metal site 3 is the site where monovalent cations bind and activate FBPase (X-ray analysis has shown that metal site 3 is the only site specific for K^+, Tl^+, and presumably NH_4^+ ions), whereas metal site 1 is the site where monovalent cations bind and inhibit the enzyme. In this case, monovalent cations may either replace the divalent cation at this site or coexist with the divalent cation in this negatively charged pocket. Our kinetic data suggest that the former is true for K^+ inhibition, and the latter may be true for Li^+ inhibition. These arguments are supported by the finding that the effects of monovalent cations on FBPase are size dependent (Nakashima & Tuboi, 1976).

The kinetic data shown in Fig. 2 also suggest that with Glu280, binding of K^+ occurs at site 3. When Glu280 was mutated to glutamine, K^+ lost the ability to activate the enzyme and became a noncompetitive inhibitor with respect to Mg^{2+}. Presumably K^+ can only bind to metal site 1 of the mutant form of FBPase and inhibit the enzyme. This is deduced from crystal structure data (Villeret et al., 1995) showing that Li^+ binds to site 1, and the kinetic findings in this report showing that Li^+ is a noncompetitive inhibitor with respect to Mg^{2+}. Metal site 1 of the mutant enzyme may not be as well defined as it is in the wild-type enzyme so that K^+

may coexist with Mg^{2+} at this site and thus play a role as a noncompetitive inhibitor. Nakashima and Tuboi (Nakashima & Tuboi, 1976) found that the effects of monovalent cations on FBPase were size dependent. Li^+ is the only monovalent cation known to have only inhibitory effects on FBPase. From Fig. 3, it can be seen that, at saturating levels of FBP, Li^+ is a noncompetitive inhibitor with respect to Mg^{2+}. Possibly due to the geometric constraints at the active site of the enzyme and the ionic radius of the cation, Li^+ may coexists with Mg^{2+} at site 1, and inhibits the enzyme by disturbing the geometry of Mg^{2+} and its ligands. Villeret et al. (1995) has shown that the guanidinium group of Arg 276 is pushed aside in the presence of K^+ ions.

The effects of monovalent cations on FBPase have long been recognized (Hubert et al., 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Marcus and Hosey, 1980; Nakashima & Tuboi, 1976), but the mechanism of action of these ions remains unknown. It is well known that monovalent cations have similar effects on Hsc70 protein (O'Brian & Mckay, 1995) and on FBPase. The difference between Hsc 70 and FBPase is that there is no positive residue at the K^+ binding site in Hsc70, whereas FBPase has Arg276 at this site. In the case of Hsc70 protein, K^+ participates directly in the hydrolysis of ATP. It facilitates the hydrolysis by either deshielding the target phosphorus atom and making it more susceptible to nucleophilic attack or by electrostatically

stabilizing the pentavalent transition state (Wilbanks & Mckay,1995). Some proteins have positive residues at their active site which play the role of monovalent cations. For example, the ε-amino group of Lys18 in actin forms a salt-bridge to oxygen of ADP (Kabsch et al., 1990), and Lys16 within the P-loop of H-ras p21 (Pai et al., 1990) has been shown to fulfill the role of K^+. However, in the case of FBPase, Arg276 and K^+ cannot replace each other, and both must be present for the enzyme to reach its optimal activity. When Arg276 was mutated to methionine, the enzyme lost greater than 99% of its activity (Zhang & Fromm, 1995), yet K^+ can still activate, but cannot completely restore the activity of the mutant FBPase (Zhang and Fromm, unpublished observations). This suggests that Arg276 plays an important role in enzyme activity. This residue is also known to be essential for Mg^{2+} sigmoidicity, and it determines the kinetic mechanism of FBPase (Zhang & Fromm, 1995).

On the basis of our kinetic data and the X-ray structure data obtained by Villeret et al. (1995), it is postulated that metal site 3 of FBPase is the monovalent cation activation site where monovalent cations such as K^+, Tl^+, or NH_4^+, together with Arg276, activate the enzyme by "deshielding" the 1-phosphoryl group of the substrate and aid in the nucleophilic attack at the phosphorus atom by OH^-. In addition, this region of FBPase is involved with Mg^{2+} ions and surrounding residues of the protein in maintaining a protein

conformation that allows the 1-phosphoryl group of FBP to achieve the optimal position required for catalysis. On the other hand, metal site 1 of the enzyme is the site responsible for monovalent cation inhibition of the enzyme. Monovalent cations inhibit FBPase at site 1 by either distorting the geometry of the active site or by retarding product release in a manner similar to the inhibitory effect of Li^+ on inositol monophosphatase (Pollack et al., 1994). The effects of monovalent cation inhibition, and/or activation of FBPase, may represent a common phenomenon associated with a number of phosphotransferase enzymes.

References

Benkovic, P.A., Caperelli, C.A., deMaine, M.M., and Benkovic, S.J. (1978) Proc. Natl. Acad. Sci. U. S. A. **75**, 2185-2189.

Benkovic, S.J., and deMaine, M.M. (1982) Adv. Enzymol. Relat. Areas Mol. Biol. **53**, 45-82.

Burton, V.A., Chen,M., Ong,W.C., Ling,T., Fromm,H.J., and Stayton,M.M. (1993) Biochem. Biophys. Res. Commun. **192**, 511-517.

Burton, V.A., Chen, M., Ong, E.E., Ling, T., Fromm, H.J., and Stayton, M.M. (1993) Biochem. Biophys. Res. Commun. **192**, 511-517.

Chen,L., Remesh,H., Chen,M., and Fromm,H.J. (1993) Arch. Biochem. Biophys. **307**, 350-354.

Colombo, G., and Marcus, F. (1973) J. Biol. Chem. **248**, 2743-2745.

Fromm, H.J. (1975) Initial Rate Enzyme Kinetics, pp. 295-306, Spring-Verlag, Berlin.

Gomori, G. (1943) J. Biol. Chem. **148**, 139-149.

Hers, H.G., and Hue, L. (1983) Annu. Rev. Biochem. **52**, 617-653.

Hers, H.G., and Van Schaftingen, E. (1982) Biochem. J. **206**, 1-12.

Hubert, E., Villanueva, J., Gonzalez, A.M., and Marcus, F. (1970) Arch. Biochem. Biophys. **138**, 590-597.

Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F., and Holmes, K.C. (1990) Nature **347**,37-44.

Ke, H., Thorpe, C.M., Seaton, B.A., Marcus, F., and Lipscomb, W.N. (1989) Proc. Natl. Acad. Sci. U. S. A. **86**, 1475-1479.

Ke, H., Zhang, Y., and Lipscomb, W. N. (1990) Proc. Natl. Acad. Sci. U. S. A. **87**, 5243-5247.

Krebs, H.A. (1963) in Advances in Enzyme Regulation (Weber, G., ed) Vol. 1, pp. 385-400, Pergamon Press Ltd., London.

Liu, F., and Fromm, H.J. (1988) J. Biol. Chem. **263**, 9122-9128.

Liu, F., and Fromm, H.J. (1990) J. Biol. Chem. **265**, 7401-7406.

Marcus, F. (1975) Biochemistry **14**, 3916-3921.

Marcus, F. (1981) in The Regulation of Carbohydrate Formation and Utilization in Mammals (Veneziabe, C.M., ed.) pp. 269- 290, University Park Press, Baltimore.

Marcus, F., Edelstein, L., Reardon, I., and Heinrikson, R.L. (1982) Proc. Natl. Acad. Sci. U. S. A. **79**, 7161-7165.

Marcus, F., and Hosey, M.M. (1980) J. Biol. Chem. **255**, 2481-2486.

Nakashima, K., and Tuboi, S. (1976) J. Biol. Chem. **251**, 4315-4321.

O'Brien, M.C., and Mckay, D.B. (1995) J. Biol. Chem. **270**, 2247-2250.

Pai, E.F., Krengel, U., Petsko, G.A., Goody, R.S., Kabsch, W., and Wittinghofer, A. (1990) EMBO J. **9**, 2351-2359.

Pilkis, S.J., El-Maghrabi, M.R., and Claus, T.H. (1988) Annu. Rev. Biochem. **57**, 755-783.

Pollack, S.J., Atack, J.R., Knowles, M.R., McAllister, G., Ian Ragen, C., Baker, R., Fletcker, S.R., Iversen, L.L., and Broughton, H.B. (1994) Proc. Natl. Acad. Sci. USA **91**, 5766- 5770.

Pontremoli, S., and Traniello, S. (1975) Methods Enzymol. **42**,369-374.

Stone, S.R., and Fromm, H.J. (1980) Biochemistry, **19**, 620-625.

Tejwani, G.A., Pedrosa, F.O., Pontremoli, S., and Horecker, B.L. (1976) Arch. Biochem. Biophys. **177**, 255-264.

Van Schaffingen, E., and Hers, H.G. (1980) Biochem. Biophys.

Res. Commun. **96**, 1524-1531.

Villeret, V., Huang, S., Fromm, H.J., and Lipscomb, W.N. (1995) Proc. Natl. Acad. Sci. U. S. A. **92**, 8916-8920.

Villanueva, J., and Marcus, F. (1974) J. Biol. Chem. **249**, 745-749.

Wilbanks, S.M., and Mckay, D.B. (1995) J. Biol. Chem. **270**, 2251-2257.

Xu, G., Yu, Z., Hu, G., and Cao, H. (1993) Biochem. Biophys. Res. Commun. **194**, 1483-1490.

Zhang, R., and Fromm, H.J. (1995) Biochemistry **34**, 8190-8195.

Zhang, Y., Liang, J.-Y., Huang, S., Ke, H., and Lipscomb, W.N. (1993) Biochemistry **32**, 1844-1857.

CHAPTER 5. SITE-DIRECTED MUTAGENESIS OF THE SUBSTRATE BINDING SITE OF PORCINE FRUCTOSE-1,6-BISPHOSPHATASE[1]

A paper published in Archives of Biochemistry and Biophysics[2]

Lie-Fen Shyur[3], Rulin Zhang[3,4], and Herbert J. Fromm[3,5]

Abstract

N212, R243, Y244, y264, and K274, which are conserved in all known primery sequences of fructose-1,6-bisphosphatase (FBPase), are located in the substrate binding domain on the basis of crystal structure of the enzyme. Mutations of the five residues of porcine liver FBPases (N212A, R243M, Y244F, Y264F, and K274L) were carried out by site-directed mutagenesis. The wild-type and mutant forms of the enzyme were purified to homogeneity and characterized by initial-rate kinetics and circular dichroism spectrometry (CD). The mutants exhibited k_{cat} values that are similar to those of the wild-type enzyme. The K_m values for the substrate of the

---

[1]This research was supported in part by Research Grants NS 10546 and GM 06920 from the National Institute of Health, United State Public Health Service, and Grant MCB-9218763 from the National Science Foundation. Thia is Journal Paper J-16138 of Iowa Agriculture and Home Economics Experiment Station, Ames; Project 2575.
[2]Reprinted with permission of Archives of Biochemistry and Biophysics, 1995, **319**, 123-127.
[3]Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011
[4]Purified K274L and performed kinetic data collection for K274L, R243M, and N121A mutant enzymes.
[5]To whom correspondence should be addressed.

mutants are 6- to 44-fold higher than that of the wild-type enzyme. The K_i values for fructose-2,6-bisphosphate (F26P) and AMP of the mutants increased from 56- to 1950-fold and 12- to 27-fold, respectively, relative to those of the wild-type enzyme. The alteration of inhibition constants for both inhibitors suggest that these five active site residues are involved in the inhibition by F26P and AMP. No apparent differences in secondary structure of the wild-type and mutant forms of FBPase were observed as measured by CD. This report demonstrate that N212, R243, Y244, Y264, and K274 not only are the sites for substrate binding, but also play important role in the binding affinity of inhibitors F26P and AMP.

Introduction

Fructose-1,6-bisphosphatase (FBPase, EC. 3.1.3.11.) plays a key role in the gluconeogenesis pathway and has been studied in a number of living system (1,2). The enzyme catalyzes the hydrolysis of fructose-1,6-bisphosphate (FBP) to form fructose 6-phosphate (F6P) and inorganic phosphate (Pi). FBPase, a homotetramer (3), is known to be synergistically inhibited by AMP and fructose-2,6-bisphosphate (F26P) (4-6). These compounds are potent activators of phosphofructokinase (7) and are responsible for the coordinated regulation of glycolysis and glyconeogenesis (1,2). Because the concentration of AMP remains relatively constant in the cell due to the presence of adenylate kinase, the level of F26P, which fluctuates under

different physiological conditions, is thought to be the dynamic regulator of these two pathways.

It has long been recognized that mammalian FBPases have an absolute requirement for exogenous divalent metal ions such as Mg^{2+}, Mn^{2+}, and Zn^{2+} (1, 2). In addition, the enzyme is activated by monovalent cations, such as K^+ and NH_4^+ (8). The solution of the crystal structure of FBPase (9,10) has shown that the enzyme is a dimmer of dimmers and can exist in two different conformational states, the active R state and the inactive T state. Two binding sites for divalent metal ions have been identified as well as an allosteric site for AMP (11). Both Mg^{2+} activation and AMP inhibition are cooperative phenomena (12-17); however, only one Mg^{2+} ion has been associated with each subunit (11).

Recent studies from this laboratory (18-20) and that of Pilkis (21-23) involving site-directed mutagenesis experiments have been consistent with the x-ray diffraction data. These investigations demonstrated that k_{cat} decreased more than 99% when mutations are made in the metal binding site of FBPase (19). In addition, there is a total loss of AMP cooperativity when E29, which is associated with the AMP binding site, is mutated to glutamine (18). Divalent metal ion cooperativity can also be eliminated when G122 is mutated to alanine (20). Interestingly, the G122 residue is associated with substrate, rather than metal binding per se (11).

Little information exists on the FBPase residues that are

predicted from the crystal structure to interact with the substrate. In this report, we describe experiments on five FBPase mutants associated with the 6-phosphoryl group of FBP. Although mutation of these residues has a small effect on k_{cat}, they are important in substrate, F26P, and AMP binding.

Experimental Procedures

Materials--NADP, FBP, Fru-2,6-P_2, AMP, Hepes, and Tris were purchased from Sigma (St. Louis, MO). Glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim (Indianapolis, IN). Distilled deionized water was used in all experiments. All other reagents were of the highest purity available commercially. N212A, R243M, Y244F, Y264F, and Y258F, were obtained by site-directed mutagenesis as described elsewhere (19). The primers used for mutagenesis are:

5'-TACAGCATCGCTGAAGGCTAT-3' for N212A

5'-TACGGGGCCATGTACGTGGGC-3' for R243M

5'-GGGGCCAGGTTCGTGGGCTCC-3' for Y244F

5'-ATCTTTATGTTCCCAGCAAAC-3' for Y264F

5'-CCCAAAGGACTGTTAAGACTG-3' for K274L

The candidate bases for mutations are shown in boldface. The pET-11a expression vector carrying either the wild-type or mutant FBPase gene was transformed into a E. coli DE657 host cell, a strain deficient in the FBPase gene. Mutant forms of the enzyme were obtained in yields comparable to those of the

wild-type enzyme. Porcine liver and kidney FBPase are identical in their primary sequences (24).

Preparation and Purification of FBPases--Recombinant and mutant forms of FBPase were prepared as described elsewhere (24) and purified as follows: Cells were collected by centrifugation at 5,000 rpm at 4 ^{o}C for 10 min and washed once with 10 mM phosphate buffer, pH 7.0. The washed cell pellet was resuspended in 100 ml water containing 1 mM EDTA, 1mM PMSF, and 2.5 μg/ml leupeptin. The cells were broken by French Press under 8000-12,000 psi. The process was repeated three times. Then, 600 μl of 1 M $MgCl_2$ and 200 μl of 10 mg/ml DNAase were added to the solution. The suspension was incubated at room temperature for 15 min with occasional inversion and then centrifuged at 13,000 rpm for 1 hr. The supernatant fluid was heated at 60 ^{o}C for 3 min with vigorous shaking. The mixture was cooled to 4 ^{o}C and centrifuged at 13,000 rpm and 4 ^{o}C for 25 min. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant solution while stirring, and proteins that precipitated between 30 and 75% saturation of $(NH_4)_2SO_4$ were collected by centrifugation at 13,000 rpm and 4 ^{o}C for 15 min. The pellet was dissolved in 15 ml water containing 1 mM PMSF, 1 mM EDTA, and 2.5 μg/ml leupeptin. The solution was applied to Sephadex G-100 (3 x 60 cm) and eluted with water. The fractions with milky color were pooled and loaded onto a Hydroxyapatite (Bio-Gel HT from Bio-Rad) column (2 x 30 cm). FBPase was eluted as a single peak by a

phosphate gradient from 1 mM to 300 mM (pH 6.8). Fractions with A_{280} greater than 0.1 and specific activity greater than 20 were pooled and dialyzed against 30 mM Tri-HCl buffer (pH 7.5) before use. The FBPase was at least 95% pure as judged by SDS-PAGE. The Hydroxyapatite column was regenerated by washing with 1 M phosphate buffer (pH 6.8) and equilibrated with 1 mM phosphate buffer, pH 6.8. This method is simpler and the yield is higher compared with the method described previously (24).

Protein Assay--Total protein was measured by the Bio-Rad method with bovine serum albumin (from Sigma) as a standard.

Circular Dichroism Spectrometry--CD studies on the wild-type and mutant forms of FBPase were carried out in 5 mM Hepes buffer (pH 7.5) at room temperature in a JASCO CD spectrometer model J-710. Samples were placed in a 1-mm cuvette, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was the average of three scans and was calibrated to remove the background of the buffer and smoothed by using a program in the computer for the spectrometer.

Kinetic Studies--FBPase activity during purification and the specific activity of pure enzyme was measured by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (28). All other kinetic experiments were done using a fluorometric assay (28) at pH 7.5 (30 mM Tris-HCl buffer) and 24^{o}C. The initial-rate data were analyzed for kinetic mechanisms by using a MINITAB

language program with an α value of 2.0 (28).

Results

Purification of wild-type and mutant forms of FBPase--The wild-type and five mutant forms of FBPase were purified to near homogeneity (greater than 95% purity determined by scanning densitometry, GS 300 Hoefer Scientific Instruments) (data not show).

Initial-rate Studies--To evaluate the effects of mutations on the substrate binding site of FBPase, kinetic studies were undertaken on the wild-type and mutant forms of FBPase. The kinetic data are summarized in Table I. Most of the data in Table I were obtained by measuring the initial rate at saturating FBP or Mg^{2+} concentrations. No significant alteration in specific activity and turnover number was found by mutating the five substrate binding site residues. Mutation of R243, which was postulated to be involved in the communication between the adjacent monomers (30) caused a 4-fold decrease in the catalytic activity; however, we do not consider this alteration to be highly significant.

Substrate Dependence of Wild-type and Mutant Forms of FBPase--It has been proposed from x-ray diffraction studies that N212, R243, Y244, Y264, and K274 are located in the active site and are involved in the interaction with 6-phosphoryl group of the substrate (30). Consistent with that investigation is the observation that Km values for FBP are

Table I. Kinetic Parameters of Wild-type and Mutant Forms of FBPases

Enzyme	k_{cat} (s^{-1})	K_m(FBP) (μM)	K_i(F26P) (μM)	K_i(AMP) (μM)	K_a(Mg^{2+}) (mM^2)	Hill coef. (Mg^{2+})
Wild-type	14	4 ± 0.2	0.2 ± 0.02	21 ± 6	0.5 ± 0.2	2.0 ± 0.1
N212A	14	116 ± 19	48 ± 4	435 ± 82	0.8 ± 0.1	1.7 ± 0.1
R243M	3	67 ± 14	267 ± 21	560 ± 97	1.6 ± 0.3	1.5 ± 0.1
Y244F	13	155 ± 9	45 ± 6	406 ± 69	0.7 ± 0.3	1.3 ± 0.1
Y264F	18	21 ± 1	14 ± 4	295 ± 72	0.3 ± 0.1	1.5 ± 0.0
K274L	12	94 ± 3	469 ± 153	250 ± 49	1.1 ± 0.1	1.4 ± 0.1

increased by mutation of these five residues. The Km values of the mutant enzymes increased 6- to 44-fold compared with the Km of the wild-typeenzyme. These data suggest that these residues play a role in substrate binding.

Kinetics of F26P Inhibition--F26P, a competitive inhibitor of FBPase, competes with FBP for binding to the active site of the enzyme. Although modest alterations would be expected for the binding affinity for F26P on the basis of increased Km values for FBP of the mutants, the 50- to 1950-fold elevation in Ki values for F26P were unexpected. These results support the x-ray data, which pinpointed these residues in FBPase as being ligated to F26P, and indicated that these residues are important in allowing FBPase to discriminate between the substrate and the inhibitor. The dramatic change of Ki for F26P was also reported for K274A mutant of rat liver FBPase (22).

Kinetics of AMP Inhibition--AMP was first reported by Taketa and Pogell (17) to be a noncompetitive inhibitor of FBPase. In addition, they demonstrated that AMP exhibits sigmoidal inhibition with a Hill coefficient of 2.4. The binding cooperativity of AMP with wild-type FBPase was observed at pH 7.5 and pH 9.5 (28,31). In this study, mutation of binding site residues associated with the 6-phosphoryl group of FBP and F26P resulted in a decrease in the binding affinity of AMP to FBPase. The Ki values for AMP of the mutants increased 12- to 27-fold compared with that of the

wild-type FBPase and the five mutants gave excellent fit to the following equation with n = 2,

$$\frac{1}{v}=\frac{1}{V_m}\left[1+\frac{I^n}{K_{ii}}+\frac{K_a}{A}\left(1+\frac{I^n}{K_i}\right)\right] \quad (1)$$

where v, V_m, A, I, K_a, K_i, and K_{ii} represent initial velocity, maximal velocity, the concentration of FBP, the concentration of AMP, the Michaelis constant for FBP, the inhibition constants for AMP, respectively, and where n is the element that represents the cooperativity. These results show that the cooperative binding of AMP is not affected by mutations at the substrate binding site.

Mg^{2+} ion cooperativity--in the presence of Mg^{2+} ion, the activity of FBPase was shown to respond sigmoidally at pH 7.2 with a Hill coefficient of 2, whereas the cooperativity for Mg^{2+} is lost at pH 9.6 (12, 13). In this study, the Hill coefficients greater than 1 were obtained for all the mutant enzymes. The Ka for Mg^{2+} and K_m for FBP of these enzymes were determined by using an initial-rate equation based on stochastic data evaluation to be

$$\frac{1}{v}=\frac{1}{V_m}\left(1+\frac{K_a}{A^n}+\frac{K_b}{B}+\frac{K_{ia}K_b}{A^nB}\right) \quad (2)$$

where v, V_m, A, B, K_a, K_b, and K_{ia} represent initial velocity, maximal velocity, the concentration of free Mg^{2+}, the

concentration of free FBP, the Michaelis constant for Mg^{2+}, the Michaelis constant for FBP, and the dissociation constant for Mg^{2+}, respectively, where n represents the cooperativity for Mg^{2+} with FBPase. Small changes in K_a values for Mg^{2+} were exhibited in K274L and R243M relative to wild-type FBPase.

The kinetic data for wild-type and mutant forms of FBPase gave excellent fits to Eq.2 with n = 2. These results are consistent with the Hill coefficient of 2. On the basis of this investigation, the five substrate binding residues are not directly involved in Mg^{2+} cooperativity. Elimination of Mg^{2+} cooperativity was also observed for wild-type and mutant FBPases at pH 9.5 (data not shown).

Circular Dichroism Spectrometry--CD studies on the wild-type and mutant forms of FBPase were carried out in 5 mM Hepes buffer (pH 7.5) at room temperature in a JASCO CD spectrometer model J-710. Samples were placed in a 1-mm cuvette, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was the average of three scans and was calibrated to remove the background of the buffer and smoothed by using a program in the computer for the spectrometer.

Discussion

The findings of this investigation accord well with the reported x-ray diffraction studies (30). The residues investigated by site-directed mutagenesis are all involved in

binding to the 6-phosphoryl group of FBP or F26P. The side chain hydroxyl groups of Y244 and Y264 as well as the NH2 group of N212 are hydrogen bonded to the substrate or inhibitor. On the other hand, the guanidinium group of R243 from the adjacent chain forms a salt-bridge with the 6-phosphoryl group of the substrate or inhibitor. The $NH3^+$ group of K274 donates two hydrogen bonds to O5 and O6 atoms of the substrate and also interacts with the 2-phosphoryl group of F26P to form a salt-bridge (33).

The conserved primary sequences of FBPase has been documented in a number of eukaryotic and prokaryotic species (30). Residues 212, 243, 244, 264, and 274, are postulated to be at the active site and involved in the binding of substrate, product, and inhibitor based on the crystallographic data of pig kidney FBPase (10, 30, 33). Although four base substitutions were found in the cDNA of FBPase from pig kidney as compared with the enzyme from pig liver (24,34). In good agreement with the crystallographic studies, the replacement of N212 by alanine, R243 by methionine, Y244 by phenylalanine, Y264 by phenylalanine, and K274 by leucine results in a decrease in binding affinity for FBP relative to the wild-type enzyme.

R243 has been predicted to be involved in signal transmission between two adjacent monomers of FBPase by mutually exchanging their R243 side chains when binding substrate or F26P (30, 35). The 1100-fold increase in K_i

observed for F26P with R243M mutant enzyme can be correlated with the loss of the salt-bridge of the R243 side chain with the oxygen atom of the 6-phosphate of F26P. A 4-fold decrease in turnover number of R243M mutant suggests that R243 has a marginal effect on catalysis of FBPase.

In addition to the binding to the oxygen atom of the furanose ring and ester oxygen (O6) of FBP and F26P, K274 also interacts with the oxygen atom of 2-phosphoryl group of the inhibitor. With mutation of K274 to leucine, these interactions were abolished. This may explain the dramatic increase in K_i for F26P.

Similar alterations in K_i values for AMP and F26P for N212 and Y244 are shown in Table II. Elimination of side chain by substituting asparagine for alanine would disturb not only the binding to FBP/F26P, but also the binding to R243 from the neighboring monomer. It is not clear why mutations at the substrate binding site increase the K_i for AMP. The phenomenon of AMP/F26P synergism is well established in the case of FBPase (4-6). Because F26P binds at the active site, it is possible that the residues that binds 6-phosphoryl group of FBP or F26P communicate in some way with the AMP site which is 29 Å away (9, 10, 30). In this study, the greatest effect of the mutations was on F26P inhibition rather than on the substrate binding affinity.

References

1. Tejwani, G.A. (1983) Adv. Enzymol. Relat. Areas Mol. Biol. **54**, 121-194.
2. Benkovic, S. J., & deMaine, M. M. (1982) Adv. Enzymol. Relat. Areas Mol. Biol. **53**, 45-82.
3. Pontremoli, S., & Traniello, S. (1975) Methods Enzymol. **42**,369-374.
4. Liu, F., & Fromm, H. J. (1988) J. Biol. Chem. **263**, 9122-9128.
5. Pilkis, S. J., El-Maghrabi, M. R., & Claus, T. H. (1988) Annu. Rev. Biochem. **57**, 755-783.
6. Van Schaftingen, E., and Hers, H-G (1981) Proc. Natl. Acad. Sci. USA **78**, 2861-2863.
7. Hers, H. G., & Van Schaftingen, E. (1982) Biochem. J. **206**, 1-12.
8. Hubert, E., Villanueva, J., Gonzalez, A. M., & Marcus, F. (1970) Arch. Biochem. Biophys. **138**, 590-597.
9. Liang, J-Y., Zhang, Y., Huang, S., and Lipscomb, W.N. (1993) Proc. Natl. Acad. Sci. USA **90**, 2132-2136.
10. Ke, H., Liang, J-Y, Zhang, Y., and Lipscomb, W.N. (1991) Biochemistry **30**, 4412-4420.
11. Zhang, Y., Liang. J.-Y., & Lipscomb, W. N. (1993b) Biochem. Biophys. Res. Commun. **190**, 1080-1083.
12. Nimmo, H. G., & Tipton, K. F. (1975a) Eur. J. Biochem. **58**, 567-574.

13. Tashima, Y., and Yoshimura, N. (1975) J. Biochem. **78**, 1161-1169.

14. Sola, M., Oliver, F.J., Salto, R., Gutierrez, M., and Vargas, A.M. (1993) Intl. J. Biochem. **25**, 1963-1968.

15. Tejwani, G. A., Pedrosa, F. O., Pontremoli, S., & Horecker, B. L. (1976) Arch. Biochem. Biophys. **177**, 255-264.

16. Sarngadharan, M.G., Watanable, A., and Pogell, B.M. (1969) Biochemistry **8**, 1411-1419.

17. Taketa, K., and Pogell, B.M. (1965) J. Biol. Chem. **240**, 651-662.

18. Chen, M., Chen, L., and Fromm, H.J. (1994) J. Biol. Chem. **269**, 5554-5558.

19. Chen, L., Remesh, H., Chen, M., & Fromm, H. J. (1993) Arch. Biochem. Biophys. **307**, 350-354.

20. Zhang, R., Chen, L., Villeret, V., and Fromm, H.J. (1995) J. Biol. Chem. **270**, 54-58.

21. El-Maghrabi, M.R., and Pilkis, S.J. (1993) J. Biol. Chem. **268**, 9466-9472.

22. El-Maghrabi, M.R., and Pilkis, S.J. (1992) J. Biol. Chem. **267**, 6526-6530.

23. Gidh-Jain, M., and Lipscomb, W.N. (1994) J. Biol. Chem. **269**, 27732-27738.

24. Burton, V. A., Chen, M., Ong, W. C., Ling, T., Fromm, H. J., & Stayton, M. M. (1993) Biochem. Biophys. Res. Commun. **192**, 511-517.

25. Nakashima, K., & Tuboi, S. (1976) J. Biol. Chem. **251**, 4315-4321.

26. Bradford, M.M. (1976) Anal. Biochem. **72**, 248-254.

27. Ulm, E., and Benkovic, S.J. (1975) Methods Enzymol. **42**, 369-374.

28. Liu, F., and Fromm, H.J. (1990) J. Biol. Chem. **265**, 7401-7406.

29. Siano, D.B., Zyskind, J.W., and Fromm, H.J. (1975) Arch. Biochem. Biophys. **170**, 587-600.

30. Ke, H., Thorpe, C.M., Seaton,B.A., and Lipscomb, W.N. (1989) J. Mol. Biol. **212**, 513-539.

31. Stone, S.R., and Fromm, H.J. (1980) Biochemistry, **19**, 620-625.

32. Leatherbarrow, R.J. (1987) ENZYFITTER A Non-linear regression Data Analysis Program for the IBMPC, pp.13-75, Elsevier Science,Cambridge/united Kingdom.

33. Liang, J-Y, Huang, S., Zhang, Y., Ke, H., and Lipscomb, W.N. (1992) Proc. Natl. Acad. Sci. USA **89**, 2404-2408.

34. William, M.K., and Kantrowitz, E.R. (1992) Proc. Natl. Acad. Sci. USA **89**, 3080-3082.

35. Ke, H., Zhang, Y., Liang, J-Y, and Lipscomb, W.N. (1991) Proc. Natl. Acad. Sci. USA **88**,2989-2993.

CHAPTER 6. MUTAGENESIS AND MODELING OF METAL SITE 3 OF PORCINE LIVER FRUCTOSE-1,6-BISPHOSPHATASE[1]

A paper submitted to Biochemistry for publication

Rulin Zhang[2,3] Richard B. Honzatko[3],

and Herbert J. Fromm[3,4,5]

Abstract

Metal site 3 of porcine liver fructose-1,6-bisphosphatase (FBPase) is defined by R276, E280, and 1-phosphate of the substrate [Villeret, V., Huang, S., Fromm, H.J., and Lipscomb, W.N. (1995) Proc. Natl. Acad. Sci. U.S.A., **92**, 8916-8920]. Two mutants, R276M and E280Q, were prepared by site-directed mutagenesis. Kinetic effects of monovalent cations on wild-type and the mutant enzymes were studied. With the wild-type enzyme, K^+ activates the enzyme at low concentration (K_m = 17 mM) and inhibits the enzyme at high concentrations (K_{IK+} = 68 mM) [Zhang, R, Villeret, V., Lipscomb, W. N., & Fromm, H. J. (1996) Biochemistry 35, 3038-3043]. Na^+ inhibits the enzyme

---

[1]This research was supported in part by Research Grant NS 10546 from the National Institutes of Health, United State Public Health Service, and Grant MCB-9218763 from the National Science Foundation. This is Journal Paper J-16751 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Project 3191.

[2]Primary researcher and author under the supervision of Herbert J. Fromm.

[3]Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011.

[4]To whom all correspondence should be addressed.

[5]Major professor.

slightly, whereas Li^+ is a stronger linear noncompetitive inhibitor with respect to Mg^{2+} and the substrate. The inhibition effects of these monovalent cations, however, are abolished when R276 is mutated to methionine. On the other hand, the activation effect of K^+ is enhanced (600% activation relative to 250% activation with the wild-type enzyme). In the case of E280Q mutant, K^+ inhibits the enzyme activity. The results in this report suggest that R276 is essential for monovalent cation inhibition and E280 is essential for monovalent cation activation. On the basis of this and previous reports, it is postulated that a specific bonding network is responsible for the enzyme to perform optimal catalysis and communication among metal sites 1 and 3 as well as the allosteric AMP site, which is distal to the metal binding sites. The findings of this report, not only contribute to an understanding of the kinetics and mechanisms of monovalent cation action, but also provide an understanding of signal transmission in FBPase.

Introduction

Phosphoryl transfer enzymes form one of the largest families of biological catalysts (Knowles, 1980). Most of these enzymes, if not all, are activated and/or inhibited by monovalent cations, yet the mechanism of the action of monovalent cations is not fully understood. Fructose-1,6-bisphosphatase (FBPase, EC 3.1.3.11), which plays an important

role in the regulation of gluconeogenesis (Krebs, 1963; Marcus, 1981; Hers & Hue, 1983; Pilkis et al., 1988), belongs to this family. It catalyzes the hydrolysis of fructose 1,6-bisphosphate (FBP) to form fructose 6-phosphate (F6P) and inorganic phosphate. The enzyme is a homotetramer with a subunit molecular mass of 37 kDa (Marcus et al., 1982; Burton et al., 1993). It requires a divalent cation, such as Mg^{2+}, Mn^{2+}, or Zn^{2+}, for activity (Gomori, 1943; Benkovic & deMaine, 1982). The saturation curves of these divalent cations show sigmoidicity at neutral pH (7.5), whereas they are hyperbolic at pH 9.6 (Nimmo & Tipton, 1975a,b). The sigmoidicity is enhanced by K^+ (Hubert et al., 1970). AMP and fructose 2,6-bisphosphate (Fru-2,6-P_2) are the two synergistic inhibitors that control the activity of this enzyme (Hers & Schaftingen, 1982; Liu & Fromm, 1990).

Since the finding of the activation effect of K^+ on pyruvate kinase (Boyer et al., 1942), monovalent cations have been shown to regulate many enzymes (Suelter, 1970). Some of these enzymes require a monovalent cation for activity, others do not. The roles of monovalent cations in enzyme catalysis and/or regulation are not clear. FBPase, like all other phosphotransferases, can be affected by monovalent cations (Marcus, 1975; Hubert et al., 1970; Villanueva & Marcus, 1974; Colombo & Marcus, 1973; Nakashima & Tuboi, 1976; Marcus & Hosey, 1980; Zhang et al., 1996). Among the monovalent cations studied, K^+, NH_4^+, and Tl^+ are the best activators,

whereas Li^+ is an inhibitor; however, how these monovalent cations affect the activity of FBPase is not fully understood. The activation effect of K^+ was thought to be the result of reversing the inhibition caused by high concentrations of divalent metal ions (Tejwani et al., 1976) or from the binding of K^+ at an allosteric site (Xu et al., 1993). Marcus (1975) reported that activation of FBPase by K^+ could be abolished by modifying an arginyl residue at the active site. These findings indicated that K^+ ion probably binds at the active site. Hubert et al. (1970) reported that K^+ ions could affect the sigmoidicity of the saturation curves of divalent cations.

Recently, x-ray diffraction studies (Villeret et al., 1995) demonstrated that K^+ and Tl^+ ions bind at three metal sites located at the active site of FBPase (Fig.1). Two sites, defined as sites 1 and 2, correspond to the divalent metal sites previously defined (Zhang et al., 1993a). The third site is thought to be specific for K^+ and Tl^+, is 3.4 Å away from site 1, and is defined by E280, R276, and the 1-phosphoryl group of the substrate (Villeret et al., 1995). Therefore, E280 and R276 are thought to be critical to the kinetics of monovalent cation action with FBPase. It is known that E280 and R276 are important for the activity of FBPase (Chen et al., 1993; Zhang & Fromm, 1995). Furthermore, R276 determines Mg^{2+} cooperativity and the kinetic mechanism (Zhang & Fromm, 1995), and E280 is essential for K^+ activation (Zhang et al., 1996). To gain insight into the effects and mechanisms

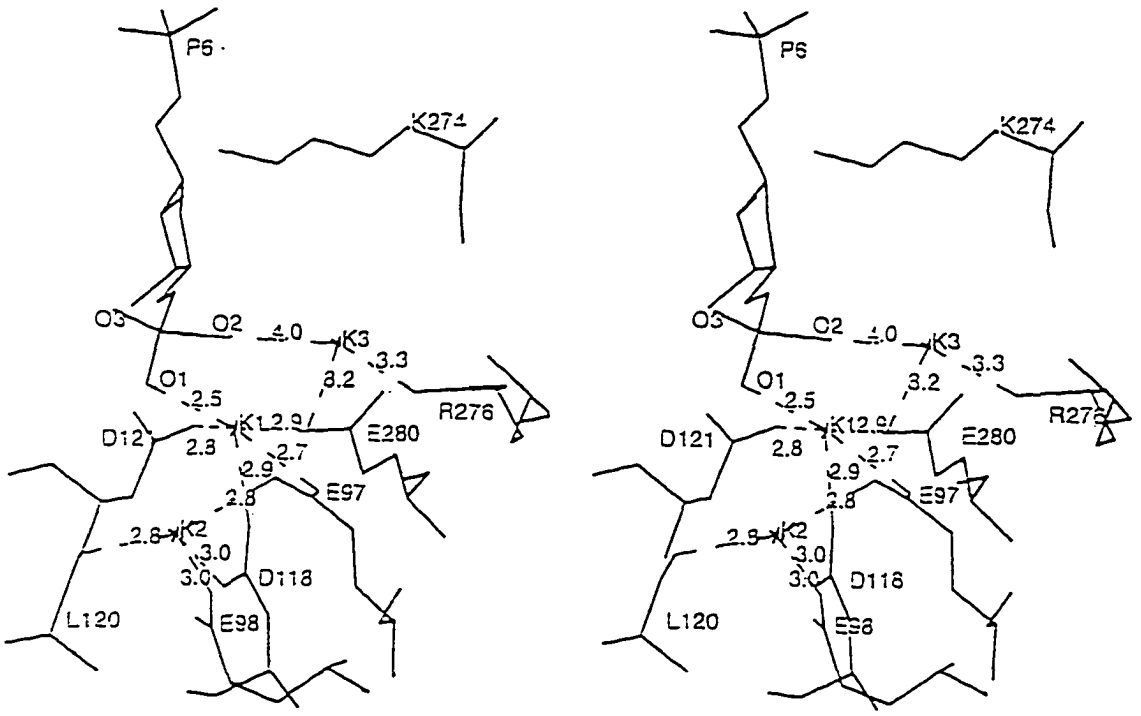


Fig. 1 Stereo model of the metal binding sites as observed in the crystal structure of FBPase (Villeret et al., 1995)(Protein Data Base entry 1FPI). K1, K2, K3 are potassium ions at the three metal binding sites. Site 3 is 3.4 Å from site 2, K1 and K2 are 3.9 Å apart. Distances between K^+ ions and their ligands are indicated. Site 1 and site 2 are specific for divalent cations, site 1 is also the inhibition site for monovalent cations, site 3 is the activation site specific for monovalent cations.

of monovalent cations on FBPase, and to understand how monovalent cations can affect the sigmoidicity of the saturation curves of divalent cations, how monovalent cations coordinate with divalent cations and the surrounding residues of the enzyme to orient the substrate and permit the enzyme to achieve optimal catalytic activity, and how different sites of the enzyme can communicate with each other, we performed kinetic studies to investigate the effects of monovalent cations on wild-type and the mutant FBPases. K^+ is known to be the best monovalent cation activator of FBPase (Hubert et al., 1970); however, it also inhibits the enzyme at high concentrations (Zhang et al., 1996). Na^+ has a minor effect, and Li^+ is an inhibitor of FBPase (Hubert et al., 1970; Zhang et al., 1996). Here, we report that mutation of R276 to methionine totally abolishes the inhibition effects of monovalent cations, while the activation effect of K^+ is enhanced. On the other hand, K^+ becomes an inhibitor when E280 is replaced by glutamine. With the wild-type enzyme, Li^+ is a noncompetitive inhibitor with respect to Mg^{2+} and the substrate. On the basis of our results and previous reports (Zhang et al., 1996; Villeret et al., 1995; Zhang et al., 1993a; Hubert et al., 1970), modeling of the two mutant FBPase were performed to explain the molecular mechanisms of action of these monovalent cations and their interactions with amino acid residues at the active site of FBPase.

Experimental Procedures

Materials--NADP, FBP, Fru-2,6-P_2, AMP, Hepes, and Tris were purchased from Sigma (St. Louis, MO). Glucose-6-phosphate dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim (Indianapolis, IN). Distilled deionized water was used in all experiments. All other reagents were of the highest purity available commercially. Mutants of recombinant porcine liver FBPase, R276M and E280Q, were obtained by site-directed mutagenesis as described elsewhere (Chen et al., 1993). The pET-11a expression vector carrying either the wild-type or mutant FBPase gene was transformed into a E. coli DE657 host cell, a strain deficient in the FBPase gene. Mutant forms of the enzyme were obtained in yields comparable to those of the wild-type enzyme. Porcine liver and kidney FBPase are identical in their primary sequences (Burton et al., 1993).

Preparation and Purification of FBPases--Recombinant and mutant forms of FBPase were prepared as described elsewhere (Burton et al., 1993) and purified as follows: Cells were collected by centrifugation at 5,000 rpm at 4 ^{o}C for 10 min and washed once with 10 mM phosphate buffer, pH 7.0. The washed cell pellet was resuspended in 100 ml water containing 1 mM EDTA, 1mM PMSF, and 2.5 μg/ml leupeptin. The cells were broken by French Press under 8000-12,000 psi. The process was repeated three times. Then, 600 μl of 1 M $MgCl_2$ and 200 μl of 10 mg/ml DNAase were added to the solution. The suspension

was incubated at room temperature for 15 min with occasional inversion and then centrifuged at 13,000 rpm for 1 hr. The supernatant fluid was heated at 60 ^{o}C for 3 min with vigorous shaking. The mixture was cooled to 4 ^{o}C and centrifuged at 13,000 rpm and 4 ^{o}C for 25 min. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant solution while stirring, and proteins that precipitated between 30 and 75% saturation of $(NH_4)_2SO_4$ were collected by centrifugation at 13,000 rpm and 4 ^{o}C for 15 min. The pellet was dissolved in 15 ml water containing 1 mM PMSF, 1 mM EDTA, and 2.5 µg/ml leupeptin. The solution was applied to Sephadex G-100 (3 x 60 cm) and eluted with water. The fractions with milky color were pooled and loaded onto a Hydroxyapatite (Bio-Gel HT from Bio-Rad) column (2 x 30 cm). FBPase was eluted as a single peak by a phosphate gradient from 1 mM to 300 mM (pH 6.8). Fractions with A_{280} greater than 0.1 and specific activity greater than 20 were pooled and dialyzed against 30 mM Tri-HCl buffer (pH 7.5) before use. The FBPase was at least 95% pure as judged by SDS-PAGE. The Hydroxyapatite column was regenerated by washing with 1 M phosphate buffer (pH 6.8) and equilibrated with 1 mM phosphate buffer, pH 6.8. This method is simpler and the yield is higher compared with the method described previously (Burton et al., 1993).

Protein Assay--Total protein was measured by the Bio-Rad method with bovine serum albumin (from Sigma) as a standard.

Circular Dichroism Spectrometry--CD studies on the wild-type and mutant forms of FBPase were carried out in 5 mM Hepes buffer (pH 7.5) at room temperature in a JASCO CD spectrometer model J-710. Samples were placed in a 1-mm cuvette, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was the average of three scans and was calibrated to remove the background of the buffer and smoothed by using a program in the computer for the spectrometer.

Kinetic Studies--FBPase activity during purification and the specific activity of pure enzyme was measured by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (Pontremoli & Traniello, 1975). All other kinetic experiments were done using a fluorometric assay (Liu & Fromm, 1990) at pH 7.5 (30 mM Tris-HCl buffer) and 24^{o}C. The initial-rate data were analyzed for kinetic mechanisms by using a MINITAB language program with an α value of 2.0 (Liu & Fromm, 1990).

Results

Enzyme Quality--The purity of wild-type and mutant FBPases were evaluated by SDS-PAGE. The proteins were greater than 95% pure by using the criterion of electrophoresis. Also, no discernible degradation of the proteins was observed (data not shown).

Secondary Structure Analysis--The secondary structures of recombinant wild-type and the mutant of FBPases were analyzed

by CD spectrometry. The purpose of this study was to determine whether localized or global structural alterations were induced in the mutant. The CD spectral data showed that the spectrum of the mutants were essentially superimposable on that of the wild-type enzyme (data not shown). These results suggest that no major conformational changes occurred in the mutant FBPases using CD as a criterion of secondary protein structure.

Effects of K^{+} on Wild-type and the mutant FBPases--Fig. 2 shows the activation effects of K^{+} on wild-type and the mutant FBPases at saturating FBP (20 μM for wild-type enzyme and 50 μM for the mutant enzymes) and Mg^{2+} (5 mM for wild-type, 10 mM for R276M, and 20 mM for E280Q). From Fig. 2, it can be seen that K^{+} can activate wild-type enzyme about 2.5-fold. This result is consistent with previous reports (Hubert et al., 1970; Colombo & Marcus, 1973; Marcus & Hosey, 1980; Nakashima & Tuboi, 1976). However, K^{+} can activate R276M about 6-fold. In addition, it is saturated at about 150 mM in the case of wild-type FBPase, whereas the activity of the mutant enzyme continues to increase until the K^{+} concentration reaches 300 mM. It is believed that the activity of the mutant enzyme declines above 300 mM K^{+} due to the effect of ionic strength rather than saturation by K^{+}. This argument is supported by the fact that Hepes-Na^{+} buffer can cause the activity of wild-type FBPase to decrease when the buffer concentration is greater than 250 mM (data not shown). From

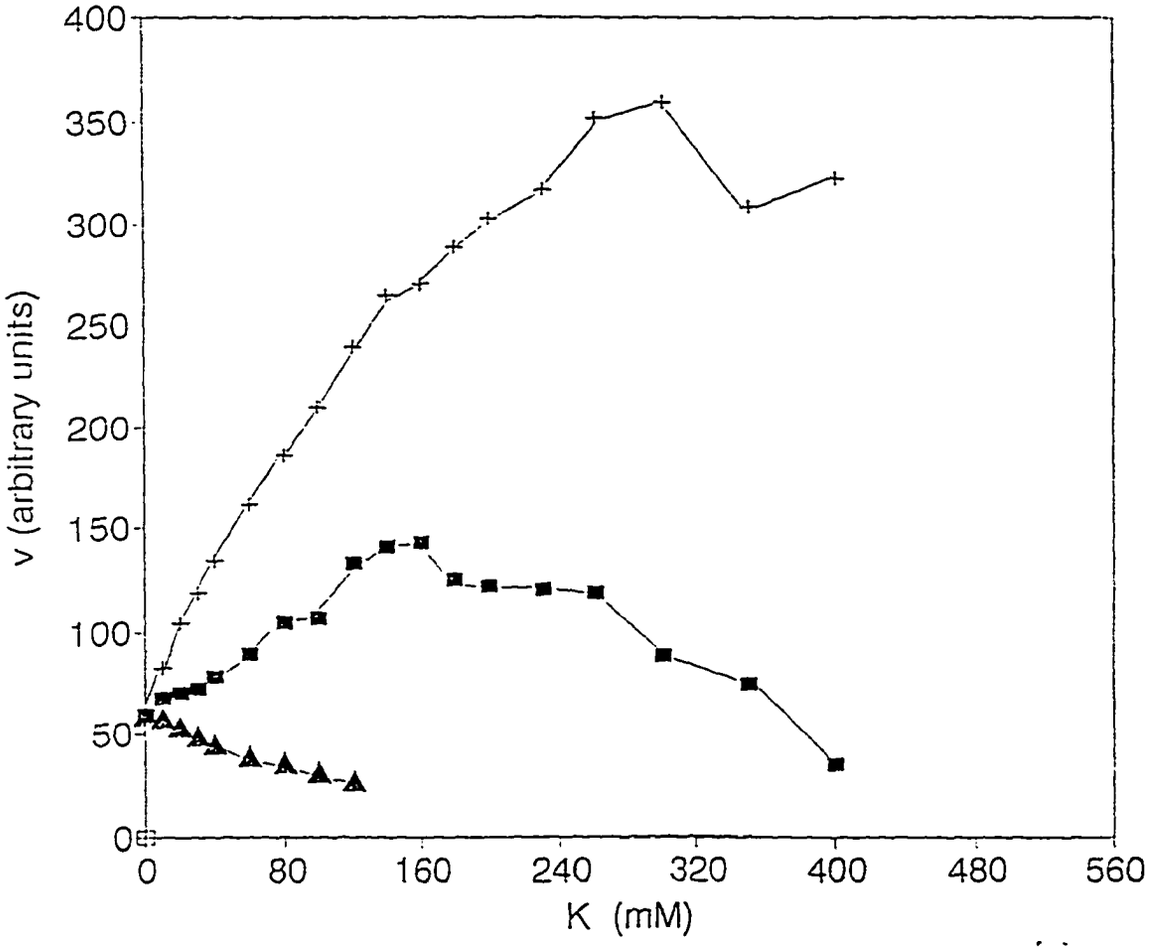


Fig. 2 Plot of initial velocity in arbitrary fluorescent units against K^+ concentration with wild-type (■), R276M (+), and E280Q (▲) FBPases. 0.34 µg wild-type, 53 µg R276M, and 450 µM E280Q FBPases were used in the assays.

Fig.2, it can also be seen that K^+ inhibits E280Q FBPase.

It has been shown (Zhang et al., 1996) that K^+ plays a dual role in affecting the activity of wild-type FBPase. It can activate and inhibit the enzyme in a concentration-dependent manner. However, the activation effect of K^+ on FBPase was lost when E280 was mutated to glutamine (Fig. 2)(Zhang et al., 1996). On the other hand, the inhibition effect of K^+ on this enzyme was totally abolished when R276 was mutated to methionine. These results are shown in Fig. 3. Fig. 3 illustrates the effects of K^+ on the kinetics of Mg^{2+} activation of mutant FBPase at saturating levels of FBP (50 μM). From Fig. 3, it can be seen that, when 1/v vs. $1/[Mg^{2+}]$ is plotted at different fixed concentrations of K^+, the lines are linear and parallel to each other, and the intercepts of the lines on 1/v axis decrease as the concentrations of K^+ increases. The data in Fig. 3 fit well to eq 1. The "Goodness of Fit" was 3.0%.

$$\frac{1}{v}=\frac{1}{V_m}\left[1+\frac{K_a}{A}+\frac{k_b}{B}\right] \quad (1)$$

where v, A, and B represent initial velocity, concentration of Mg^{2+}, and concentration of K^+, respectively; V_m is the maximum velocity in the presence of K^+; K_a and K_b are the activation constants for Mg^{2+}(1.4 mM) and K^+(93 mM), respectively. Eq 1 is obtained from eq 2 (Zhang et al., 1996) by noting that Mg^{2+} sigmoidicity is lost (the A term in eq 2 becomes first power) when R276 is mutated to methionine (Zhang & Fromm, 1995), and

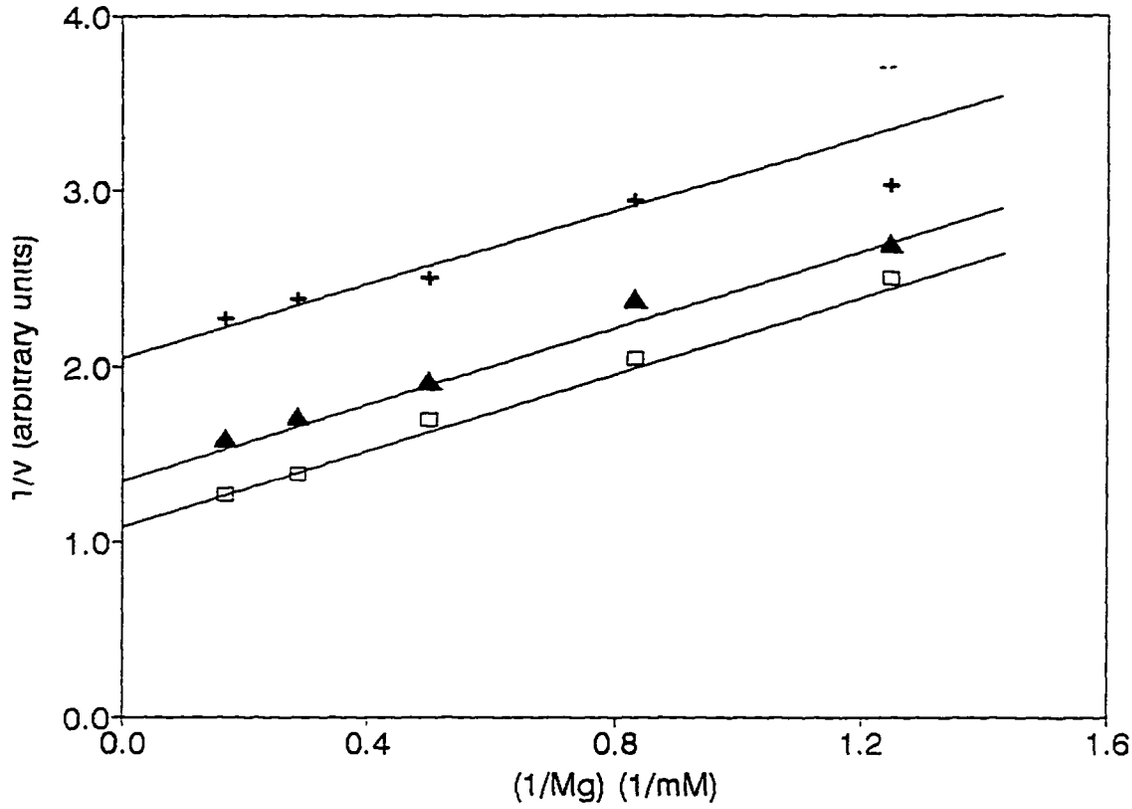


Fig. 3 Plot of reciprocal of initial velocity in arbitrary fluorescent units against reciprocal of [Mg^{2+}] at different concentrations of K^+ with R276M mutant FBPase. The concentrations of K^+ are 50 mM(■), 100 mM (+), 150 mM (▲). The lines are theoretical and based on eq 1. All the points are experimentally determined.

that the inhibitory effect of K^+ with the mutant enzyme is abolished (B/K_{IB} and B/A^2 terms are omitted). Also, in eq 2, $k_{21} >> k_{26}$, (for details of the assumptions, see Zhang et al., 1996). Thus, in eq 1, $V_m = k_{25}E_o$, $K_a = k_{18}/k_{15}k_{23}k_{25}$, $K_b = k_{22}/k_{21}$.

$$\frac{k_{25}E_o}{v} = 1 + \frac{k_{22}/k_{21}}{k_{26}/k_{21}+B} + \frac{k_{17}/k_{25}}{k_{15}k_{23}}\left(\frac{k_{18}/k_{17}+B}{A^2}\right)\left(1+\frac{B}{K_{IB}}\right) \quad (2)$$

Effects of Na^+ on wild-type and mutant FBPases--Fig. 4 illustrates the effects of Na^+ on wild-type and mutant forms of FBPase at saturating concentrations of FBP (20 μM for wild-type enzyme and 50 μM for R276M enzyme) and Mg^{2+} (5 mM for wild-type and 10 mM for R276M enzyme). From Fig. 4, it can be seen that Na^+ slightly activates the wild-type FBPase at low levels, and inhibits above 70 mM; however, the inhibitory effect is not observed up to 350 mM in the case of the mutant enzyme. The enzyme activity decreases at Na^+ concentrations above 350 mM. This is probably due to the effect of ionic strength rather than Na^+ inhibition per se. Na^+ inhibits E280Q mutant enzyme slightly (data not shown).

Effects of Li^+ on Wild-type and R276M mutant FBPases--It is well known that Li^+ is an inhibitor of FBPase (Hubert et al., 1970; Nakashima & Tuboi, 1976; Zhang et al., 1996). Fig. 5 depicts the effects of Li^+ on wild-type and R276M mutant FBPases in the presence or absence of saturating K^+ (150 mM for wild-type and 250 mM for the mutant enzyme). Mg^{2+} and

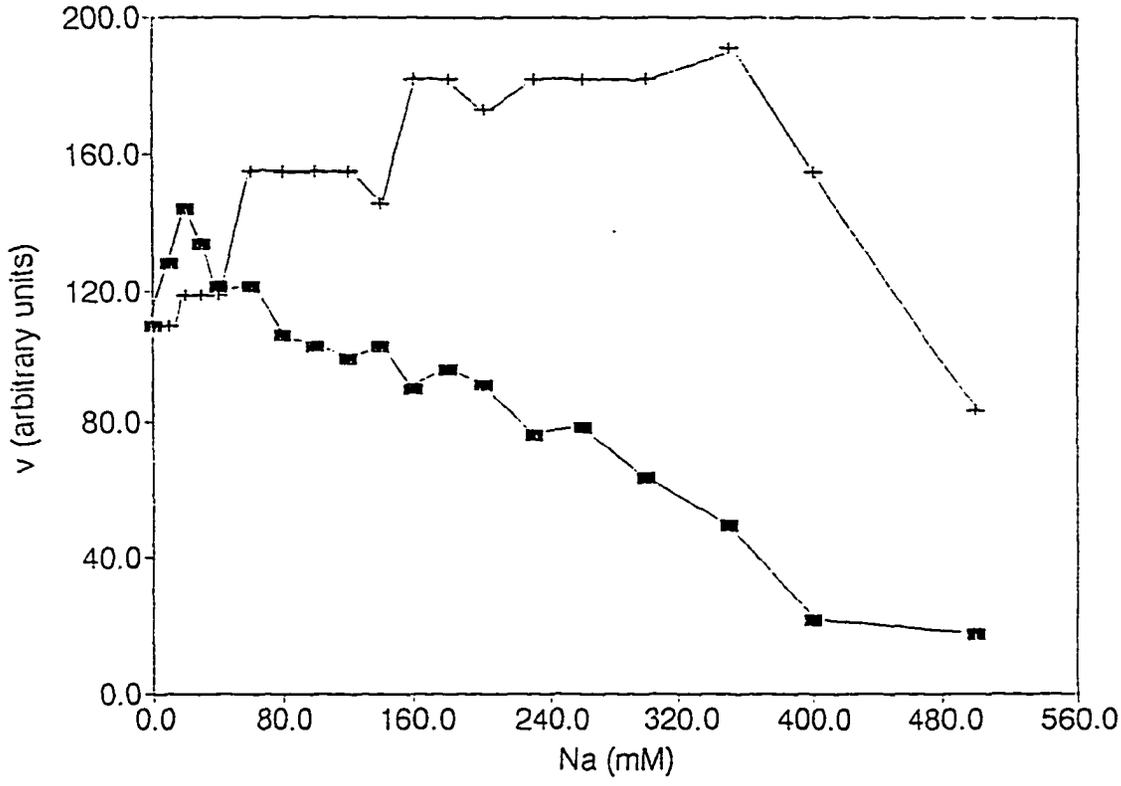


Fig. 4 Plot of initial velocity in arbitrary fluorescent units against Na^+ concentrations with wild-type (■) and R276M (+) mutant FBPases. 0.34 µg wild-type and 53 µg mutant FBPases were used in the assays.

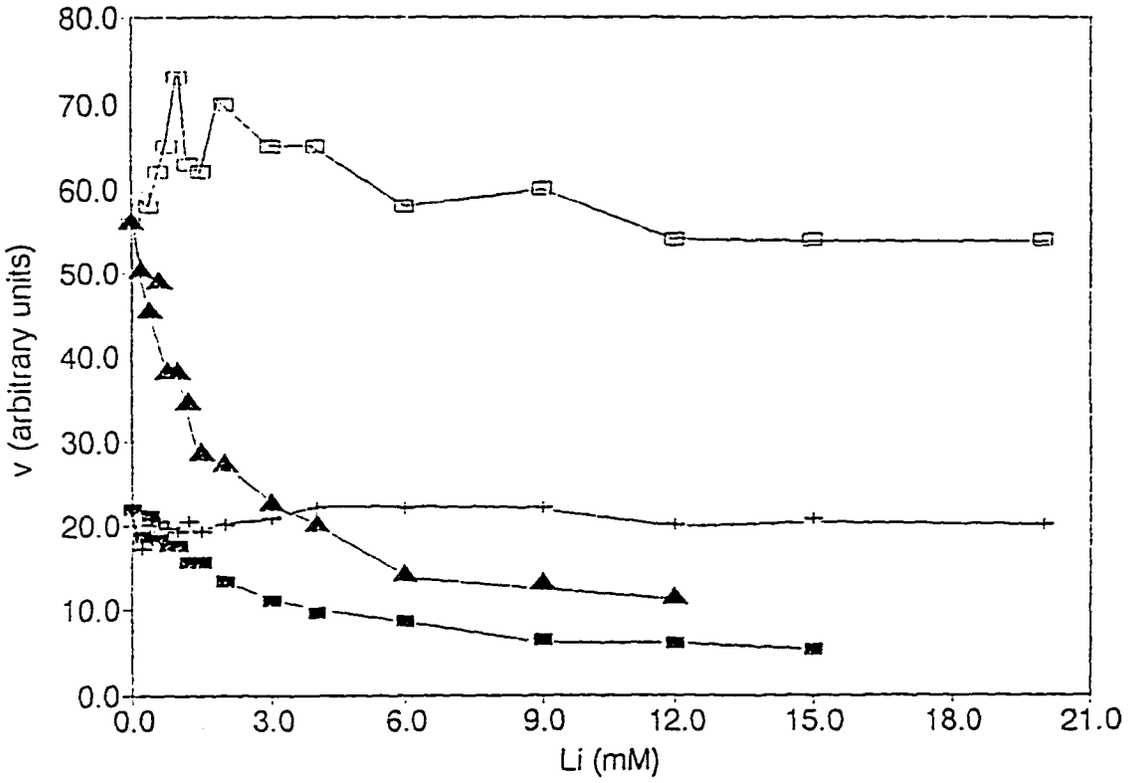


Fig. 5 Plot of initial velocity in arbitrary fluorescent units against Li^+ concentrations in the presence of 150 mM K^+ with wild-type FBPase (▲); in the absence K^+ with wild-type FBPase (■); in the presence of 250 mM K^+ with R276M mutant FBPase (□); in the absence of K^+ (+). 0.34 µg wild-type and 53 µg mutant FBPases were used in the assays.

FBP are at saturating levels in all experiments. From Fig. 5, it can be seen that, in either the presence or absence of saturating K^+, Li^+ shows strong inhibition with wild-type FBPase; however the inhibitory effects are lost with the mutant enzyme. Since Li^+ inhibits wild-type enzyme activity, its effect on E280Q mutant enzyme was not tested.

It is known that in the case of the wild-type enzyme, Li^+ is a noncompetitive inhibitor of Mg^{2+} with an inhibition constant of approximately 0.20 mM in the absence of K^+ (Zhang et al., 1996). It was found that, even in the presence of saturating (150 mM) K^+, Li^+ is still a noncompetitive inhibitor with respect to Mg^{2+} (K_i = 0.95 mM). This result is shown in Fig. 6. From Fig. 6, it can be seen that when 1/v vs. $1/[Mg^{2+}]$ is plotted at different fixed concentrations of Li^+, a family of lines is obtained that converge in the second quadrant. The data in Fig. 6 fit well to a conventional noncompetitive model with a "Goodness of Fit" of 5.0%, but do not fit to other models such as competitive or uncompetitive inhibition.

Li^+ is also found to be a noncompetitive inhibitor (K_i = 8.1 mM) with respect to K^+ at saturating concentrations of FBP (20 μM) and Mg^{2+} (5 mM). A noncompetitive inhibition pattern was obtained when 1/v was plotted versus $1/K^+$ (data not shown). The results indicate that Li^+ and K^+ are not mutually exclusive in their binding; i.e., both cations can bind to the enzyme at the same time.

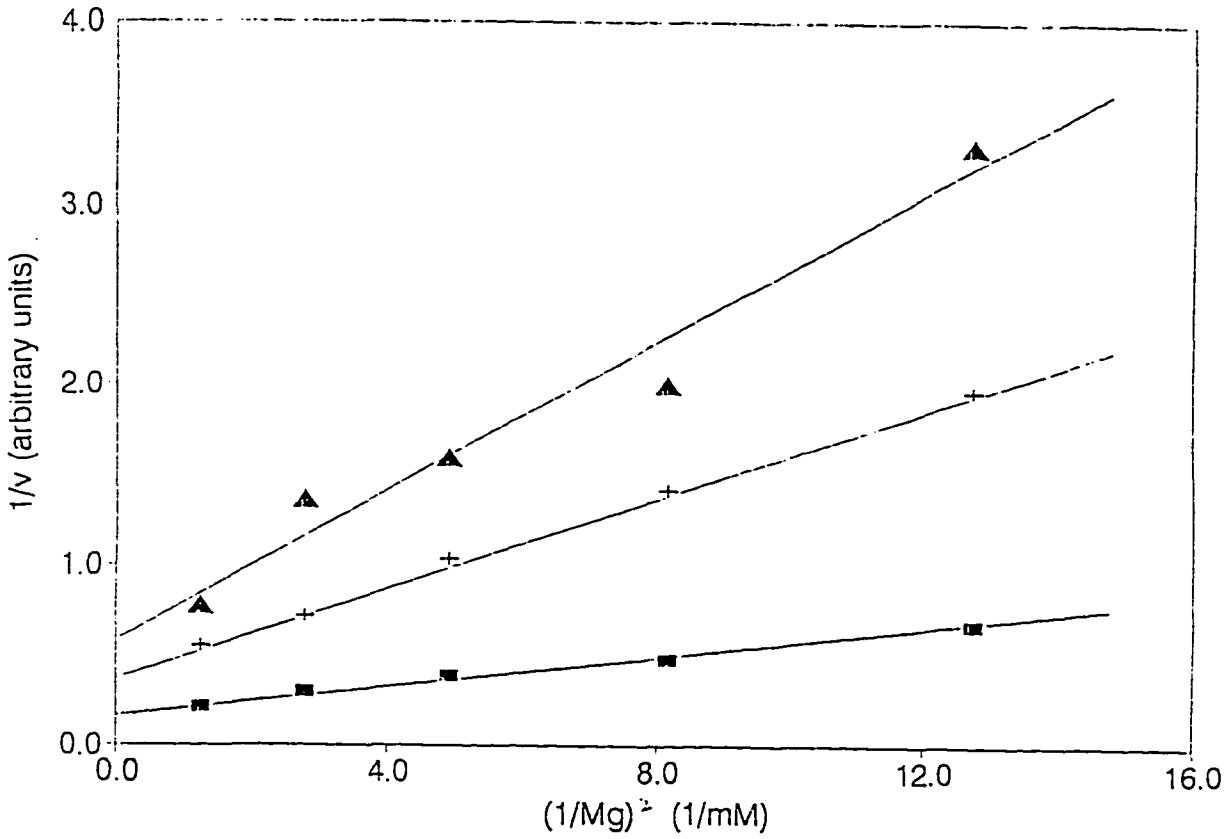


Fig. 6 Plot of reciprocal of initial velocity in arbitrary fluorescent units against reciprocal of $[Mg^{2+}]^2$ at different concentrations of Li^+ with wild-type FBPase. The concentrations of Li^+ are 0 mM (■), 2 mM (+), 4 mM (▲). The lines are theoretical and based on a conventional noncompetitive model. All the points are experimentally determined.

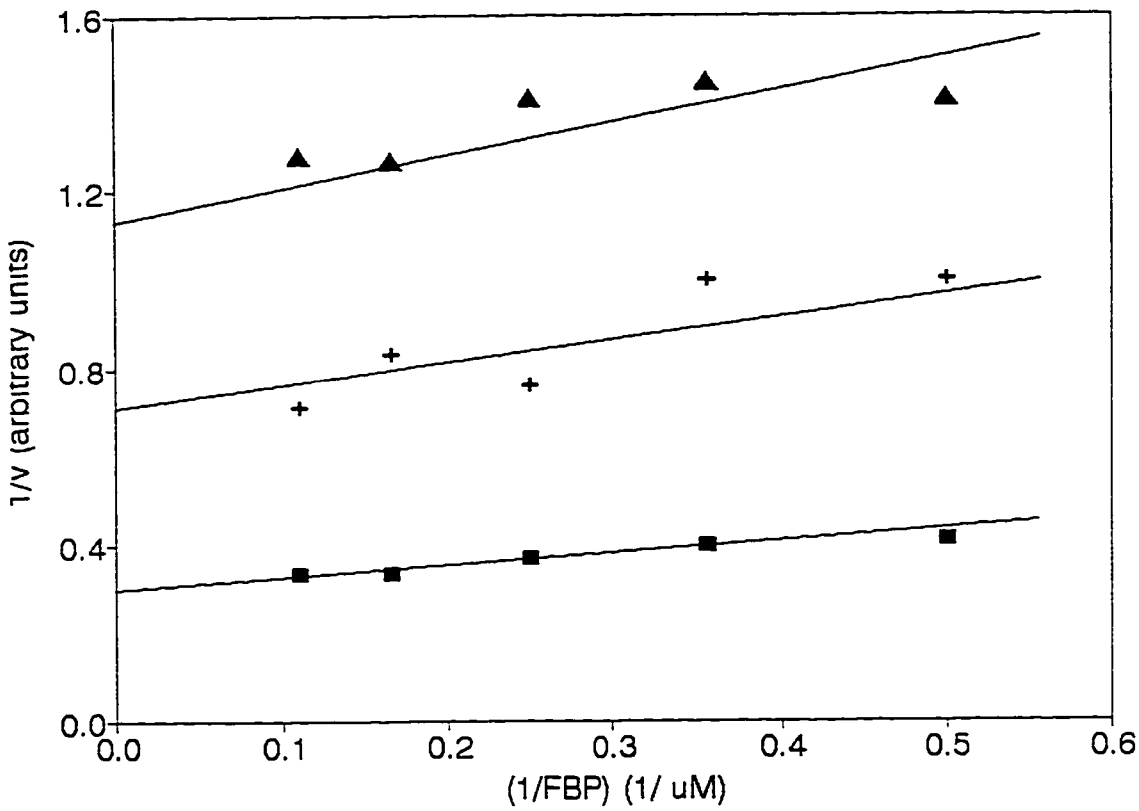


Fig. 7 Plot of reciprocal of initial velocity in arbitrary fluorescent units against reciprocal of [FBP] at different concentrations of Li^+ with wild-type FBPase. The concentrations of Li^+ are 0 mM (■), 3 mM (+), 6 mM (▲). The lines are theoretical and based on a conventional noncompetitive model. All the points are experimentally Inositol determined.

monophosphatase has a secondary structure similar to FBPase, and the effects of metal activation/inhibition on the two enzymes are similar (Zhang et al., 1993b). It is known that Li^+ is a noncompetitive inhibitor of Mg^{2+} (similar to FBPase), but it is an uncompetitive inhibitor of the substrate (Hallcher & Sherman, 1980). To evaluate the mechanism of Li^+ inhibition on FBPase, Li^+ inhibition relative to FBP was also studied. The data are shown in Fig. 7. From Fig. 7, it can be seen that Li^+ is a noncompetitive inhibitor of FBP with a K_i of 3.4 mM. The data in Fig. 7 fit well to noncompetitive model with a "Goodness of Fit" of 4.4%. The data do not fit to competitive or uncompetitive inhibition models.

Discussion

Each subunit of FBPase has three metal binding sites (Fig. 1). Sites 1 and 2 are specific for divalent metal ions such as Mn^{2+} or Zn^{2+}; however, only one Mg^{2+} binds to FBPase, and that occurs exclusively at site 1 (Zhang et al., 1993a; Benkovic & deMaine, 1982). Site 3 is specific for monovalent cations such as K^+ or Tl^+ (Villeret et al., 1995), but Li^+ binds only at site 1. Site 3 is defined by E280, R276, and the 1-phosphoryl group of the substrate analog α-methyl-fructofuranoside-1,6-bisphosphate (Villeret et al., 1995). Therefore, E280 and R276 are thought to be two critical residues in monovalent cation activation.

It is known that E280 is essential for K^+ activation, and

it is also believed that metal site 1 is the inhibition site and metal 3 the activation site for monovalent cations (Zhang et al., 1996). K^+ loses its ability to activate the enzyme and becomes a noncompetitive inhibitor with respect to Mg^{2+} at saturating FBP when E280 is mutated to glutamine. From Fig. 8, it can be seen that when E280 is changed to glutamine, the residues which define the metal sites are not affected. Metal site 1, however, relocates slightly, and it is not as well defined as is wild-type. This might allow monovalent cation to "coexist" with Mg^{2+} at site 1 to inhibit the enzyme by distorting the geometry at that region. The results in this report also demonstrate that the monovalent cations used in this study; i.e., K^+, Na^+, Li^+, lost their inhibitory effects when R276 was mutated to methionine. On the other hand, the activation effect of K^+ is enhanced by the mutation. R276, a highly conserved residue, helps determine the kinetic mechanism and Mg^{2+} sigmoidicity and is critical for enzyme activity (Zhang & Fromm, 1995).

Questions that arise from these studies are: a) How does R276 affect the kinetics of activation and/or inhibition of metal ions including divalent and monovalent cations? b) How does this residue determine the kinetic mechanism and contribute to catalysis? c) What are the mechanisms of monovalent cation action and how are they coordinated with divalent cations and the surrounding residues of the enzyme during catalysis? It is thought that the results in this

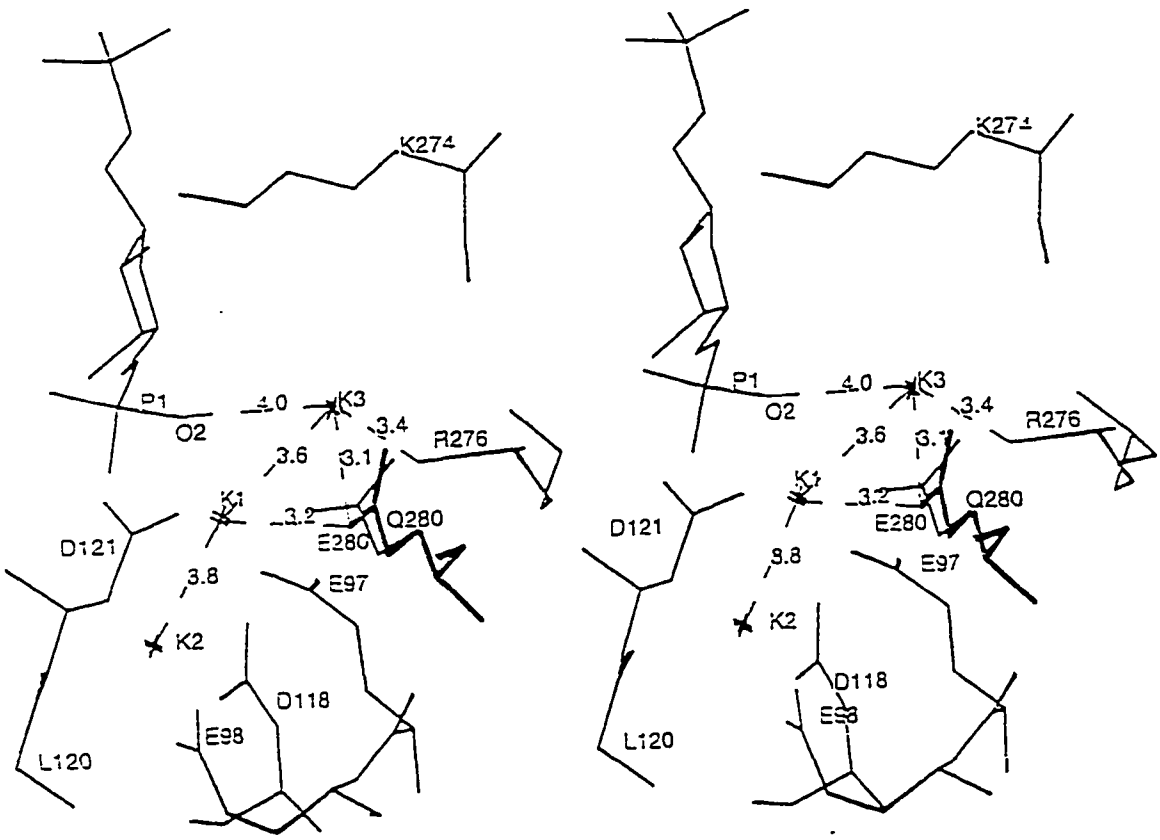


Fig. 8 Stereo model of the metal binding sites of FBPase as defined in the crystal structure (Villeret et al., 1995)(Protein Data Base entry 1FPI) and the substitution of glutamine at position 280. For clarity only the distances which are different from those in Fig. 1 are labeled. Energy minimization was performed for the mutant model. The ligands which define the metal sites are not affected by the mutation. K1 and K3 are deviated slightly from their original positions. The distance between K1 and K3 is 3.6 instead of 3.4 Å, K1 and K2 are 3.8 Å. The distance between K1 and the amine group of E280 side chain is 3.2 Å. Site 1 is not as well defined as is wild-type when E280 is replaced by glutamine.

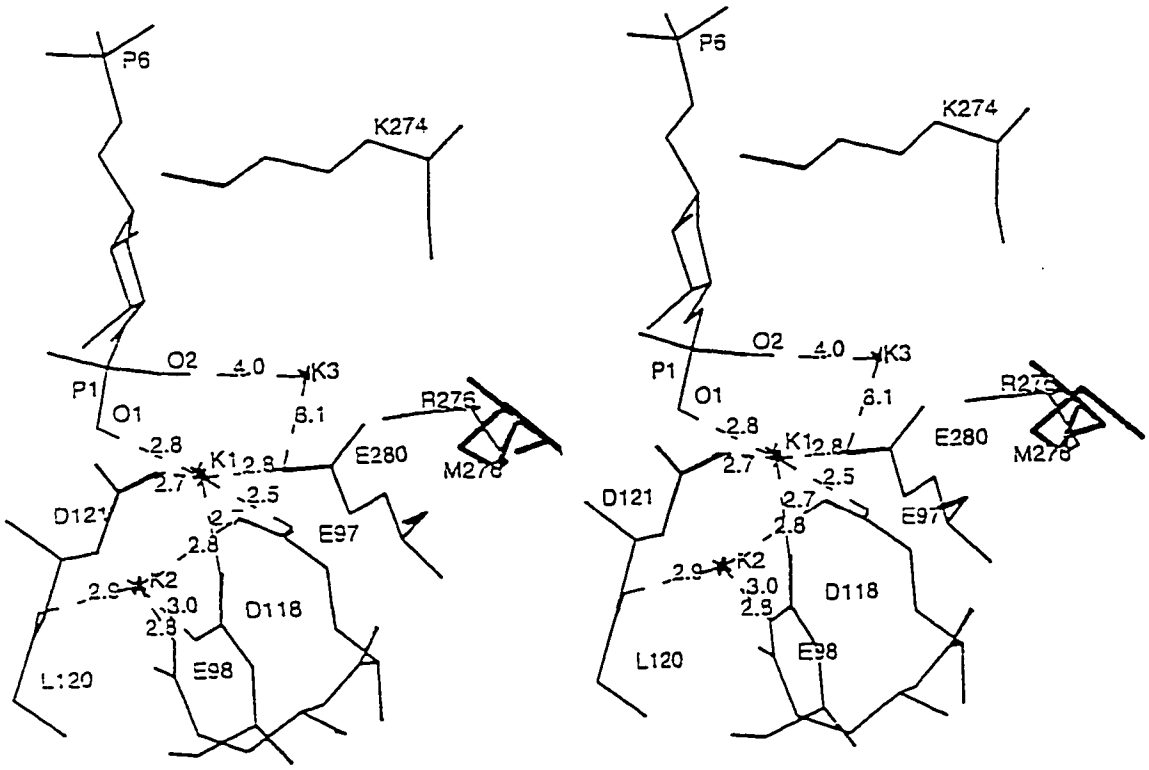


Fig. 9 Stereo model of the metal binding sites of FBPase as defined in the crystal structure (Villeret et al., 1995) (Protein Data Base entry 1FPI) and the substitution of methionine at position 276. Energy minimization was performed for the mutant model. The ligands which define the metal sites are not affected by the mutation. K1 is deviated slightly from its original position. The average distance between K1 and its ligands in the energy minimized mutant model is 0.1 Å shorter compared to that of wild-type model, this indicates that Site 1 is better defined when R276 is substituted by methionine.

report, together with those of previous studies (Hubert et al., 1970; Marcus, 1975; Zhang et al., 1993a; Villeret et al., 1995; Zhang & Fromm, 1995; Zhang et al., 1996), constitute parts of the answers to these questions.

The findings in this and previous reports suggest that FBPase has unique signal transmission pathways that allow signals to be transmitted from one site to another. It is believed that R276 can affect metal site 1, although it does not define site 1. This is based on the fact that Li^+ and Mg^{2+} bind at site 1 (Zhang et al., 1993a; Villeret et al., 1995) and that replacement of arginine residue 276 by methionine can abolish Li^+ inhibition (Fig. 5) and Mg^{2+} sigmoidicity (Zhang & Fromm, 1995). However, how R276 affects metal site 1 is not clear. It is known that R276 forms a salt bridge with the 1-phosphoryl group of the substrate, but this salt bridge no longer exists in the presence of Mg^{2+} because the divalent cation pulls the 1-phosphate group towards the divalent metal binding site (Zhang et al., 1993a). It is believed that the role of K^+ is to replace the lost salt bridge between R276 and the 1-phosphoryl group of the substrate. This salt bridge is required for the enzyme to achieve optimal activity. Thus, R276 or its replacement at site 3, K^+, can affect metal site 1 by transmitting information to the 1-phosphate of the substrate and then to metal site 1. Because E280 defines sites 1 and 3, it is not unreasonable to suggest that R276 may also affect site 1

through this bonding network to E280 and then to site 1 and, thus, affect Li^+ and/or Mg^{2+} binding. The data in Fig. 5 suggest that Li^+ cannot bind at site 1 when R276 is mutated to methionine. From Fig. 9, it can be seen that metal site 1 is better defined relative to wild-type enzyme, so that monovalent cations cannot go to site 1 to inhibit the enzyme. Besides, monovalent cations can easily go to site 3 when the positive charge of R276 side chain is removed. Zhang & Fromm (1995) reported that Mg^{2+} loses its sigmoidal kinetics with this mutant FBPase. The origin of Mg^{2+} sigmoidicity is not known. It may arise from cooperative binding of Mg^{2+} between subunits, within a subunit, or it may be a result of a steady-state kinetic mechanism. Interruption of signal transmission and/or the change of kinetic mechanism by the mutation might be the cause of the loss of Mg^{2+} sigmoidal kinetics. Inasmuch as Mg^{2+} and AMP are mutually exclusive in their binding to FBPase (Liu & Fromm, 1990), metal site 1 and the allosteric AMP binding site can communicate with each other, probably through the movement of the peptide chain and/or conformational changes of the enzyme. Therefore, a signal at site 3 can be transmitted through site 1 to the allosteric AMP site. Indeed, K^+ can affect the inhibition constant of AMP (Hubert et al., 1970).

FBPase has R276 as a positive residue at metal site 3, but it also needs K^+ for optimal activity. K^+ may function as a bridge to connect R276 and the 1-phosphate of the substrate.

The main role of R276 is believed to coordinate with divalent cations and the surrounding residues of the enzyme through the K^+ bridge to orient the substrate to an optimal position for catalysis, whereas the positive charge of R276 plays a minor role in catalysis. This argument is supported by the results that show that mutation of this residue to methionine leads to a 99.6% loss of activity and that K^+ can restore very little of the lost activity. On the other hand, K^+ is required for ATPase activity of Hsc 70 protein, which does not have a positive residue at the K^+ binding site. K^+ participates directly in the hydrolysis of ATP by stabilizing the pentavalent transition state (O'Brien & Mckay, 1995). Some other proteins, such as actin (Kabsch et al., 1990) and H-ras p21 (Pai et al., 1990) have one positive residue to fulfill the role of K^+. Inositol monophosphatase shares a similar secondary structure with FBPase (Zhang et al., 1993b). It also requires divalent cations for activity and is inhibited by Li^+. Li^+ has been found to be a noncompetitive inhibitor of Mg^{2+} and an uncompetitive inhibitor of the substrate (Hallcher & Sherman, 1980). With FBPase, Li^+ is not only a noncompetitive inhibitor of Mg^{2+} (Fig. 6), but also a noncompetitive inhibitor of K^+ (not shown) and the substrate (Fig. 7). Therefore, the mode of Li^+ action on these two phosphotransferases seems to be different. In the case of myo-inositol-1-phosphatase, Li^+ inhibits the enzyme by retarding product release (Pollack et al., 1994); however, in

the case of FBPase, Li^+ can bind to the enzyme at the same time that Mg^{2+} and K^+ bind. It is believed that Li^+ inhibits the enzyme by distorting the geometry at the active site, and a specific network formed by monovalent cations, divalent cations, and the catalytically important residues at the active site is required for the enzyme to achieve optimal activity.

On the basis of this report and previous studies, it is postulated that a specific network of enzyme residues and metal ions at the active site is required for the enzyme to achieve optimal activity. Li^+ inhibits the enzyme by distorting this network. On the other hand, K^+ may function as a bridge to connect R276 and the substrate as well as E280. This bridge is required for R276 to coordinate with divalent cations and other catalytically important residues to orient the substrate to a position required for optimal catalysis. Through this bridge, R276 may pass its action to metal site 1 and affect Li^+ inhibition and the sigmoidal kinetics observed with Mg^{2+}.

References

Benkovic, S. J., & deMaine, M. M. (1982) Adv. Enzymol. Relat. Areas Mol. Biol. **53**, 45-82.

Boyer, P. D., Lardy, H. A., & Phillips, P. H. (1942) J. Biol. Chem. **146**, 673-682.

Burton, V. A., Chen, M., Ong, W. C., Ling, T., Fromm, H. J., & Stayton, M. M. (1993) Biochem. Biophys. Res. Commun. **192**, 511-517.

Chen, L., Remesh, H., Chen, M., & Fromm, H. J. (1993) Arch. Biochem. Biophys. **307**, 350-354.

Colombo, G., & Marcus, F. (1973) J. Biol. Chem. **248**, 2743-2745.

Gomori, G. (1943) J. Biol. Chem. **148**, 139-149.

Hallcher, L. M., & Sherman, W. R. (1980) J. Biol. Chem. **255**, 10896-10901.

Hers, H. G., & Hue, L. (1983) Annu. Rev. Biochem. **52**, 617-653.

Hers, H. G., & Van Schaftingen, E. (1982) Biochem. J. **206**, 1-12.

Hubert, E., Villanueva, J., Gonzalez, A. M., & Marcus, F. (1970) Arch. Biochem. Biophys. **138**, 590-597.

Kabsch, W., Mannherz, H. G., Suck, D., Pai, E. F., & Holmes, K. C. (1990) Nature **347**,37-44.

Knowles, J. R. (1980) Annu. Rev. Biochem. **49**, 877-919.

Krebs, H. A. (1963) in Advances in Enzyme Regulation (Weber, G., ed) Vol. 1, pp. 385-400, Pergamon Press Ltd., London.

Liu, F., & Fromm, H. J. (1990) J. Biol. Chem. **265**, 7401-7406.

Marcus, F. (1975) Biochemistry **14**, 3916-3921.

Marcus, F. (1981) in The Regulation of Carbohydrate Formation and Utilization in Mammals (Veneziabe, C.M., ed.) pp.269-290, University Park Press, Baltimore.

Marcus, F., Edelstein, L., Reardon, I., & Heinrikson, R.L. (1982) Proc. Natl. Acad. Sci. U. S. A. **79**, 7161-7165.

Marcus, F., & Hosey, M. M. (1980) J. Biol. Chem. **255**, 2481-2486.

Nakashima, K., & Tuboi, S. (1976) J. Biol. Chem. **251**, 4315-4321.

Nimmo, H. G., & Tipton, K. F. (1975a) Eur. J. Biochem. **58**, 567-574.

Nimmo, H. G., & Tipton, K. F. (1975b) Eur. J. Biochem. **58**, 575-585.

O'Brien, M. C., & Mckay, D. B. (1995) J. Biol. Chem. **270**, 2247-2250.

Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) EMBO J. **9**, 2351-2359.

Pilkis, S. J., El-Maghrabi, M. R., & Claus, T. H. (1988) Annu. Rev. Biochem. **57**, 755-783.

Pollack, S. J., Atack, J. R., Knowles, M. R., McAllister, G., Ian Ragen, C., Baker, R., Fletcker, S. R., Iversen, L. L., & Broughton, H. B. (1994) Proc. Natl. Acad. Sci. USA **91**, 5766- 5770.

Pontremoli, S., & Traniello, S. (1975) Methods Enzymol. **42**,369-374.

Suelter, C. H. (1970) Science **168**, 789-795.

Tejwani, G. A., Pedrosa, F. O., Pontremoli, S., & Horecker, B. L. (1976) Arch. Biochem. Biophys. **177**, 255-264.

Villeret, V., Huang, S., Fromm, H. J., & Lipscomb, W. N. (1995) Proc. Natl. Acad. Sci. U. S. A. **92**, 8916-8920 .

Villanueva, J., & Marcus, F. (1974) J. Biol. Chem. **249**, 745-749.

Xu, G., Yu, Z., Hu, G., & Cao, H. (1993) Biochem. Biophys. Res. Commun. **194**, 1483-1490.

Zhang, R., & Fromm, H. J. (1995) Biochemistry **34**, 8190-8195.

Zhang, Y., Liang, J.-Y., Huang, S., Ke, H., & Lipscomb, W. N. (1993a) Biochemistry **32**, 1844-1857.

Zhang, Y., Liang. J.-Y., & Lipscomb, W. N. (1993b) Biochem. Biophys. Res. Commun. **190**, 1080-1083.

Zhang, R., Villeret, V., Lipscomb, W. N., & Fromm, H. J. (1996) Biochemistry **35**, 3038-3043.

CHAPTER 7. SITE-DIRECTED MUTAGENESIS AT THE C1C2 (C3C4) INTERFACE OF PORCINE LIVER FRUCTOSE-1,6-BISPHOSPHATASE AFFECT THE ENZYME'S AFFINITIES FOR LIGANDS, BUT DO NOT ABOLISH THE SYNERGISM BETWEEN AMP AND FRUCTOSE-2,6-BISPHOSPHATE[1]

A paper submitted to Archives of Biochemistry and Biophysics for publication

Rulin Zhang[2,3], Lie-Fen Shyur[3], and Herbert J. Fromm[3,4]

Abstract

Site-directed mutagenesis of amino acid residues at C1C2 (C3C4) interface in the active site domain of porcine liver fructose-1,6-bisphosphatase were carried out on the basis of crystal structure of the enzyme [Xue, Y., Huang, S., Liang, J.-Y[6]., Zhang, Y., and Lipscomb, W.N. (1994) Proc. Natl. Acad. Sci. USA 91, 12482-12486]. Mutant FBPases, S123A, D127T, C128G, R243M, and Y258F, were purified to homogeneity and characterized by circular dichroism spectrometry (CD) and initial-rate kinetics. There were no discernible differences between the secondary structures of the wild-type and the

---

[1]This research was supported in part by Research Grant NS 10546 from the National Institutes of Health, United State Public Health Service, and Grant MCB-9218763 from the National Science Foundation. This is Journal Paper J-xxxxx of the Iowa Agriculture and Home Economics Experiment Station, Ames, Project 3191.

[2]Primary researcher and author under the supervision of Herbert J. Fromm.

[3]Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011.

[4]To whom all correspondence should be addressed.

mutant enzymes on the basis of CD data. The mutations do not significantly affect the k_{cat} of the enzyme and the Hill coefficient for Mg^{2+}. The synergism between AMP and fructose-2,6-bisphosphate (F26P) were not abolished with all the mutant enzymes. The enzyme's affinities for the substrate and regulators, however, were changed in different degrees. Mutation of S123 to alanine reduced K_i for F26P to about 10-fold, K_i for AMP to about 680-fold. The K_a of this mutant enzyme for Mg^{2+}, however, increased to about 3-fold. Replacement of D127 by threonine lowered K_i for AMP to about 327-fold, but K_a for Mg^{2+} increased to about 4-fold. The most interesting mutant is C128G. The mutation caused 48-fold increase in K_m for substrate, 1680-fold increase in K_i for F26P, 34-fold increase in K_i for AMP, and 13-fold increase in K_a for Mg^{2+}. Similar results were obtained with R243M mutant. The mutation of Y258 to phenylalanine reduced the affinity for F26P to about 47-fold, and that for Mg^{2+} to about 4-fold. This report demonstrates that helix H4 and residues Y258 and R243 from adjacent chain are important in ligand binding. Also, the synergism between AMP and F26P may result from collaborative effort of many amino acid residues.

Introduction

Fructose-1,6-bisphosphatase (FBPase, EC3.1.3.11) is a key enzyme in the gluconeogenesis pathway (1-3). It catalyzes the hydrolysis of fructose-1,6-bisphosphate (FBP) to form fructose

6-phosphate (F6P) and inorganic phosphate (P_i). The reaction requires a divalent metal ion such as Mg^{2+} or Mn^{2+} for activity, and is regulated by fructose-2,6-bisphosphate (F26P) and AMP. F26P is a competitive inhibitor and AMP a noncompetitive inhibitor with respect to the substrate (4). The two inhibitors act synergistically to inhibit the enzyme (5). F26P competes with the substrate at the active site, whereas AMP binds at an allosteric site distal from the active site (6). The role of AMP is to prevent divalent metal binding to enzyme (AMP and Mg^{2+} are found to be mutually exclusive in their binding to the enzyme) (7). One of the roles of F26P is to enhance the effect of AMP by reducing the dissociation constant of AMP from the Enzyme-AMP-F26P complex (7).

FBPase is a homotetramer with D_2 symmetry. X-ray diffraction studies showed that this enzyme exists in two quaternary conformations, the active R form and the inactive T form (8-10). The binding of AMP may lock the enzyme to T state (11). It is observed that helix H4 (residues 123-127) is subject to conformational changes during catalysis. Also, H4 is better defined in the quaternary complex of Enzyme-F26P-AMP-Zn^{2+} than in the R-forms (11). In the quaternary structure, K274, S123, S124, and R243 (from adjacent chain) bind to F26P much tighter (Fig.1B) compare with that in Enzyme-F26P complex (Fig.1A). Most interestingly, Y258 from the adjacent chain turns greater than 90^{o} around C_{α}-C_{β} bond in the quaternary complex and its hydroxyl group form hydrogen

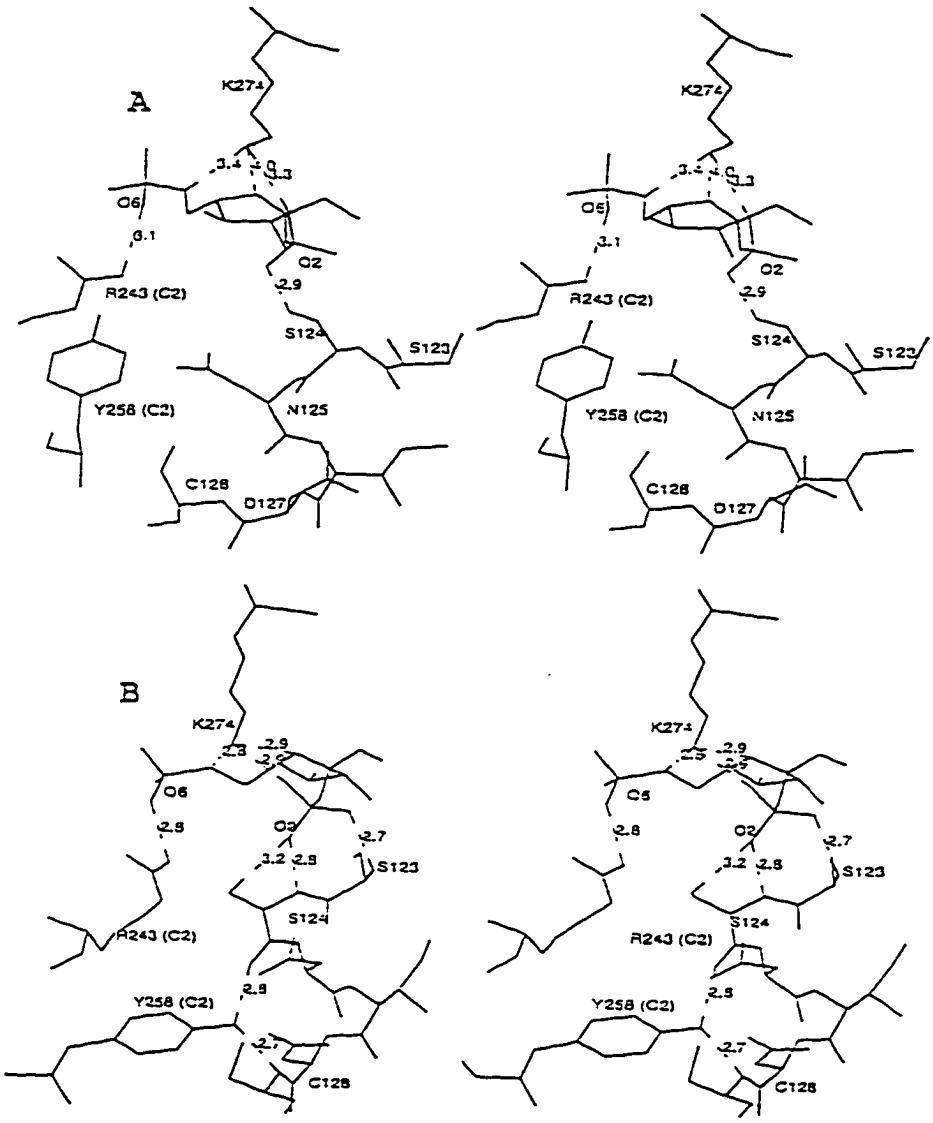


Fig.1. Stereo model of C1C2 (C3C4) interface in the active site domain of FBPase complexed with F26P (A)(6)and complexed with F26P, AMP, and Zn^{2+} (B)(11). When compared with model A, model B has a new hydrogen bond between the hydroxyl group of S123 and the oxygen of 2-phosphate, and the distance of the salt-bridge between R243 from the adjacent chain and the oxygen of the 6-phosphate changes from 3.1 Å to 2.8 Å, and the distances from the -NH3 group of K274 to oxygen atoms of the 2-phosphoryl group, the furanose ring, and the ester oxygen (O6) are shorted. Most interestingly, Y258 from the adjacent chain turns greater than 90^{o} around C_{μ}-C_{β} bond and its hydroxyl group forms hydrogen bonds to the backbone carboxyl of S124 and amide of C128 (2.8 and 2.7 Å, respectively).

bonds to the backbone carbonyl of S124 and amide of C128 (see Fig.1B). This was not observed in other T-forms. Therefore, this residue and residues of H4 are thought to be involved in the synergism between F26P and AMP.

To gain insight into the roles of H4 and residue Y258 of FBPase in ligand binding and synergism between the two inhibitors, site-directed mutagenesis were performed to substitute Y258 by phenylalanine, S123 by alanine, D127 by threonine, and C128 by glycine. The kinetic properties of these mutants were studied. We here report that mutation at the hinge region of H4 and Y258 affect the affinities of ligands. In particular, mutation of C128 to glycine caused over 1000-fold decrease in affinity for F26P, 33-fold for AMP, 48-fold for FBP, and 13-fold for Mg^{2+} ion. Synergism between AMP and F26P, however, still exists with all these mutant FBPases. Thus, it is believed that the synergism might be the collective effort of many residues of FBPase, and the enzyme uses this mechanism to avoid disruption of the synergistic regulation by a single mutation.

Experimental Procedures

Materials--NADP, FBP, Fru-2,6-P_2, AMP, Hepes, and Tris were dehydrogenase and phosphoglucoisomerase were from Boehringer Mannheim (Indianapolis, IN). Distilled deionized water was used in all experiments. All other reagents were of the highest purity available commercially. Mutants of recombinant

porcine liver FBPase, S123A, D127T, C128G, R243M, and Y258F, were obtained by site-directed mutagenesis as described elsewhere (12). The nucleotide primers used for the mutations are shown in Table I. The pET-11a expression vector carrying either the wild-type or mutant FBPase gene was transformed into a E. coli DE657 host cell, a strain deficient in the FBPase gene. Porcine liver and kidney FBPase are identical in their primary sequences (13).

Table I. Oligonucleotide Primers[a] Used in Site-Directed Mutagenesis

Mutants	Primer Sequences
S123A	5′-CGATGGA**G**CGTCGAACAT-3′
D127T	5′-GTCGAACATC**AC**CTGCCTTGTG-3′
C128G	5′-AACATCGAC**G**GCCTTGTGT-3′
R243M	5′-TACGGGGCCA**T**GTACGTGGGC-3′
Y258F	5′-CGCTGGTCT**T**TGGAGGGATC-3′

a. The bases shown in boldface are the candidates for mutations.

Preparation and Purification of FBPases--Recombinant and mutant forms of FBPase were prepared as described elsewhere (13) and purified as follows: Cells were collected by centrifugation at 5,000 rpm at 4 ^{o}C for 10 min and washed once

with 10 mM phosphate buffer, pH 7.0. The washed cell pellet was resuspended in 100 ml water containing 1 mM EDTA, 1mM PMSF, and 2.5 μg/ml leupeptin. The cells were broken by French Press under 8000-12,000 psi. The process was repeated three times. Then, 600 μl of 1 M $MgCl_2$ and 200 μl of 10 mg/ml DNAase were added to the solution. The suspension was incubated at room temperature for 15 min with occasional inversion and then centrifuged at 13,000 rpm for 1 hr. The supernatant fluid was heated at 60 ^{o}C for 3 min with vigorous shaking. The mixture was cooled to 4 ^{o}C and centrifuged at 13,000 rpm and 4 ^{o}C for 25 min. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant solution while stirring, and proteins that precipitated between 30 and 75% saturation of $(NH_4)_2SO_4$ were collected by centrifugation at 13,000 rpm and 4 ^{o}C for 15 min. The pellet was dissolved in 15 ml water containing 1 mM PMSF, 1 mM EDTA, and 2.5 μg/ml leupeptin. The solution was applied to Sephadex G-100 (3 x 60 cm) and eluted with water. The fractions with milky color were pooled and loaded onto a Hydroxyapatite (Bio-Gel HT from Bio-Rad) column (2 x 30 cm). FBPase was eluted as a single peak by a phosphate gradient from 1 mM to 300 mM (pH 6.8). Fractions with A_{280} greater than 0.1 and specific activity greater than 20 were pooled and dialyzed against 30 mM Tri-HCl buffer (pH 7.5) before use. The FBPase was at least 95% pure as judged by SDS-PAGE. The Hydroxyapatite column was regenerated by washing with 1 M phosphate buffer (pH 6.8) and equilibrated

with 1 mM phosphate buffer, pH 6.8. This method is simpler and the yield is higher compared with the method described previously (13).

Protein Assay--Total protein was measured by the Bio-Rad method with bovine serum albumin (from Sigma) as a standard.

Circular Dichroism Spectrometry--CD studies on the wild-type and mutant forms of FBPase were carried out in 5 mM Hepes buffer (pH 7.5) at room temperature in a JASCO CD spectrometer model J-710. Samples were placed in a 1-mm cuvette, and data points were collected from 200 to 260 nm in 0.5-nm increments. Each spectrum was the average of three scans and was calibrated to remove the background of the buffer and smoothed by using a program in the computer for the spectrometer.

Kinetic Studies--FBPase activity during purification and the specific activity of pure enzyme was measured by using the phosphoglucoisomerase/glucose-6-phosphate dehydrogenase coupled spectrophotometric assay (14). All other kinetic experiments were done using a fluorometric assay (4) at pH 7.5 (30 mM Tris-HCl buffer) and 24^{o}C. The initial-rate data were analyzed for kinetic mechanisms by using a MINITAB language program with an α value of 2.0 (4). The Hill coefficients for Mg^{2+} were evaluated by using ENZFIT program (15)

Results

Enzyme Quality--The purity of wild-type and mutant FBPases were evaluated by SDS-PAGE. The proteins were greater

than 95% pure by using the criterion of electrophoresis. Also, no discernible degradation of the proteins was observed (data not shown).

Secondary Structure Analysis--The secondary structures of recombinant wild-type and the mutant of FBPases were analyzed by CD spectrometry. The purpose of this study was to determine whether localized or global structural alterations were induced in the mutant. The CD spectral data showed that the spectrum of the mutants were essentially superimposable on that of the wild-type enzyme (data not shown). These results suggest that no major conformational changes occurred in the mutant FBPases using CD as a criterion of secondary protein structure.

Initial-rate Studies--Table II shows the kinetics parameters for wild-type and the mutant FBPase. The data were obtained by measuring the initial-rate at saturating Mg^{2+} or FBP concentration. From Table II, it can be seen that the K_{cat} of the mutant enzymes decreased 1.4- to about 10-fold, we do not consider these changes to be significant. In addition, the Hill coefficient of these mutant enzymes for Mg^{2+} are not changed. The affinities of the substrate and the regulators, however, are altered in different degrees by the mutations. Mutation of S123 to alanine did not change the K_m for the substrate, but lowered the K_i for F26P to about 10-fold, K_i for AMP to about 680-fold. But the K_a for Mg^{2+} increased to about 3-fold. Replacement of D127 by threonine did not affect

Table II. Kinetic Parameters of Wild-Type and Mutant Forms of FBPase

Enzyme	k_{cat} (s^{-1})	K_m(FBP) (μM)	K_i(F26P) (μM)	K_i(AMP) (μM)	(Mg^{2+}) K_a(mM)	Hill Coef.
Wild-type	20 ± 0.9	2.5 ± 0.3	0.36 ± 0.05	17 ± 5.4	0.50 ± 0.1	2.0 ± 0.09
S123A	5.3 ± 0.5	1.5 ± 0.2	0.031 ± 0.002	0.025 ± 0.01	1.6 ± 0.2	1.9 ± 0.1
D127T	14 ± 0.006	3.9 ± 0.6	0.56 ± 0.03	0.052 ± 0.01	2.1 ± 0.6	1.9 ± 0.2
C128G	4.9 ± 0.3	119 ± 21	605 ± 13	581 ± 0.92	6.5 ± 1.6	1.9 ± 0.5
Y258F	2.0 ± 0.2	1.8 ± 0.2	17 ± 0.9	13 ± 0.4	1.9 ± 0.1	1.7 ± 0.1
R243M[a]	3.3 ± 0.2	67 ± 14	267 ± 21	560 ± 97	1.6 ± 0.3	1.5 ± 0.04

a. The kinetic parameters of this mutant are taken from (16).

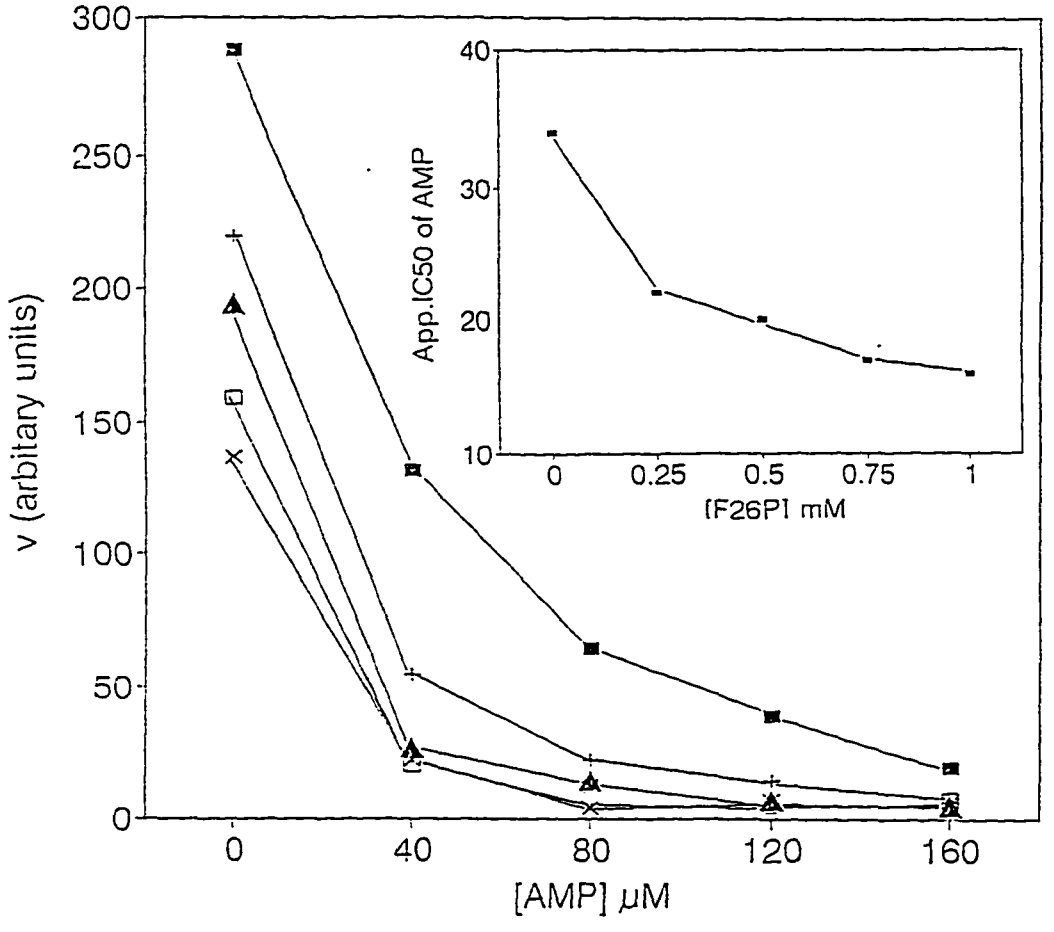


Fig. 2. Effect of F26P on the inhibition of C128G mutant FBPase by AMP. Concentration of F26P are 0mM (■), 0.25 mM (+), 0.50 mM (▲), 0.75 mM (□), and 10. mM (X). The activity was measured at 22°C in the presence of saturating FBP (0.3 mM) and Mg^{2+} (20 mM). (Inset) Effect of F26P on apparent K_i for AMP, defined as the concentration of AMP that decreased the activity to 50% of that measured in the absence of AMP.

K_m for FBP and K_i for F26P, but lowered the K_i for AMP to about 327-fold, and the K_a for Mg2+ increased to about 4-fold. The most interesting mutant is C128G. The mutation caused 48-fold increase in K_m for FBP, 1680-fold increase in K_i for F26P, 34-fold increase in K_i for AMP, and 13-fold increase in Ka for Mg^{2+}. Mutation of Y258 to phenylalanine lowered affinity for F26P to about 47-fold, and affinity for Mg^{2+} to about 4-fold. The kinetic data of R243M mutant were taken from (16). It can be seen that the mutation caused 27-fold increase in K_m for the substrate, lowered the affinity for F26P to about 742-fold, that for AMP to about 33-fold, and that for Mg2+ to about 3-fold. C128G and R243M are the only two mutants that lower the affinities for the substrate and all the regulators of the enzyme.

Effects of the Mutations on Synergism Between AMP and F26P--AMP is an allosteric inhibitor of FBPase. The inhibition of AMP is synergistically enhanced by F26P (5). X-ray diffraction data showed that binding of F26P to FBPase is enhanced in the presence of AMP. The enhancement of F26P binding resulted from the movement of H4 and the reorientation of Y258 (Fig. 1). Attempt was made to check if the mutations affected the synergism between AMP and F26P. It has been found that the synergistic effect still exist in all the mutant FBPases. Fig. 2 shows the F26P potentiation of AMP inhibition of C128G mutant FBPase (C128G mutant FBPase is taken as an example). From Fig. 2, it can be seen that the

apparent K_i value for AMP decreases as the concentration of F26P increases. Also, the apparent K_i for F26P decreases as the concentration of AMP increases (data not shown). The results indicate that the two inhibitors still act synergistically to inhibit the C128G FBPase even though the affinities of the two inhibitors were reduced by the mutation.

Discussion

X-ray diffraction data (11) showed that H4 is better defined in the Enzyme-F26P-AMP-Zn^{2+} complex compared with that in the Enzyme-F26P complex. The binding of F26P, a competitive inhibitor with respect to the substrate (4), is enhanced in the presence of AMP, an noncompetitive inhibitor binding at an allosteric site distal from the active site (6). The synergistic action between AMP and F26P has long been recognized (5-6, 11, 17-20). Thus, it is believed that residues of H4 and residues Y258 and R243 from adjacent monomer may play important roles in ligand binding and synergism between the two inhibitors. However, initial-rate studies showed that the synergistic action still exist with all the mutant FBPases. The F26P potentiation of AMP inhibition is shown in Fig. 2, which uses the data of C128G mutant as an example. The results suggest that the synergism between AMP and F26P are resulted from the collaborative effort from many amino acid residues.

S123 and D127 are at the hinge region between B3 and H4,

H4 and L6, respectively (Fig. 1). Mutation of S123 to alanine enhances the binding for both inhibitors, and change D127 to threonine also enhances the binding of AMP (Table II). It is thought that when the hydrophobicity of the side chain of the two amino acid residues are increased, H4 may be easily locked to a certain conformation which is required for the binding of the inhibitors. This is supported by the x-ray diffraction data that showed that F26P binding is enhanced when H4 is better defined (11). This is also supported by the data of C128G mutant. C128 is located at the hinge region between H4 and L6. Mutation of C128 to glycine might increase the freedom of H4. Table II shows that the enzyme's affinity for all the ligands are reduced by the mutation. These results suggest that H4 is very important for the affinities of the substrate and the regulators of FBPase. Y258 fromm the adjacent chain forms hydrogen bonds to the backbone carboxyl of S124 and amide of C128 in the structure of Enzyme-F26P-AMP-Zn^{2+} complex (Fig 1B). These hydrogen bonds may play a role to help fix H4 in a required position for ligand binding. Table II shows that replacement of Y258 by phenylalanine lowered the binding affinities of F26P and Mg^{2+}. R243 of one chain form a salt-bridge with FBP or F26P in the active site of an adjacent chain (Fig. 1). Crystal structure of R243A mutant has been solved recently (21), which showed no major changes in the organization of the active site compared with wild-type enzyme. Table II shows that when R243 was mutated

to methionine, enzyme affinities for FBP, F26P, and AMP, were reduced 27-fold, 742-fold, and 33-fold, respectively. These results suggest that R243 is also involved in the signal transmission between AMP site and the active site.

The results in this report help us to understand better the binding of the ligands and signal transmission among the ligand binding sites. Additional experiments are in progress, such as solving the crystal structure of these mutant FBPases, in order to clarify the mechanisms of signal transmission among the ligand binding sites on the same subunit and those on different subunits.

References

1. Underwood,A.H., and Newsholme,E.A. (1965) Biochem. J. **95**,767-774.
2. McGilvay,R.W. (1964) Fructose-1,6-diphosphatase and its role in Gluconeogenesis (McGilvay, R.W., and Pogell, B.M.,Eds). Port City Press Inc, Baltimore.
3. Krebs,H.A. (1963) Advances in Enzyme Regulation, Vol.1, (Weber, G., Ed.) Pergamon Press Ltd., London and New York.
4. Liu,F., and Fromm,H.J.(1990) J. Biol. Chem. **265**, 7401-7406.
5. Hers,H., and Van Schaffingen,E.(1982)Biochem. J. **206**, 1-12.
6. Liang, J.-Y., Zhang, Y., Huang, S., Ke, H., and Lipscomb, W.N. (1992) "Regulation of Protein by Ligands". Proc. Robert A. Welch Foundation Conf. Chem. Res., October 26-27, Houston, Texas.

7. Liu,F., and Fromm,H.J.(1988)J. Biol. Chem. **263**,9122-9128.

8. Ke,H., Zhang,Y., and Lipscomb,W.N. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 5243-5247.

9. Ke,H., Liang,J.Y., Zhang,Y., and Lipscomb,W.N. (1991) Biochemistry **30**, 4412-4420.

10. Zhang, Y., Liang, J-y, Huang, S., and Lipscomb, W.N. (1994) J. Mol. Biol. **244**, 609-624.

11. Xue, Y., Huang, S., Liang, J-y, Zhang, Y., and Lipscomb, W.N.(1994) Proc. Natl. Acad. Sci. USA **91**, 12482-12486.

12. Chen,L., Remesh,H., Chen,M., and Fromm,H.J. (1993) Arch. Biochem. and Biophys. **307**, 350-354.

13. Burton, V.A., Chen,M., Ong,W.C., Ling,T., Fromm,H.J., and Stayton,M.M. (1993) Biochem. Biophys. Res. Commun. **192**, 511-517.

14. Pontremodi, S., and Traniello, S. (1975) Methods Enzymol. **42**,369-374.

15. Leatherbarrow, R.J. (1987) ENZYFITTER A Non-linear Regression Data Analysis Program for the IBMPC, pp.13-75, Elsevier Science, Cambridge/United Kingdom.

16. Shyur, L., Zhang, R., Fromm, H.J. (1995) Arch. Biochem. and Biophys. **319**, 123-127.

17. Van Schaffingen,E., Hue,L., and Hers,H.G. (1980) Biochem. J. **192**, 887-895.

18. Van Schaffingen,E., Hue,L., and Hers,H.G. (1980) Biochem. J. **192**, 897-901.

19. Van Schaffingen,E., and Hers,H.G. (1980) Biochem. Biophys. Res. Commun., **96**, 1524-1531.

20. Van Schaffingen, E., and Hers, H. (1981) Proc. Natl. Acad.Sci. USA **78**, 2861-2863.

21. Stec, B., Abraham, R., Giroux, E., and Kantrowitz, E.R. (1996) Protein Science 5, 1541-1553.

CHAPTER 8. CONCLUSION REMARKS

Summary

Site-directed mutagenesis and initial-rate kinetics have been carried out to study the structure-function relationships in FBPase. X-ray diffraction data showed that the main chain NH group of the G122 residue forms a bifurcated hydrogen bond to the ester oxygen and a 1-phosphoryl oxygen of the substrate (1). It has been found that altering Gly122 to alanine caused a significant decrease in the enzyme's activity and affinity for Mg^{2+}. The K_{cat} for this mutant enzyme was only about 5% of that of wild-type fructose-1,6-bisphosphatase, and the Ka for Mg^{2+} was about 15-fold higher than that of the wild-type enzyme. The K_i for AMP was increased 77-fold in the case of the mutant enzyme; however, the Hill coefficient was unaltered. Most importantly, it was observed that replacement of Gly122 with alanine caused the total loss of cooperativity for Mg^{2+}. It is concluded that Gly122 is essential for Mg^{2+} cooperativity and important for binding of Mg^{2+} and AMP as well as for enzyme activity.

The side chain NH_3^+ group of R276 form a salt-bridge with the oxygen of 1-phosphoryl group of the substrate. Replacing Arg 276 with methionine caused a significant decrease in the enzyme's activity. The k_{cat} for this mutant enzyme was only about 0.67% of that of the wild-type enzyme. Most importantly, the mutation caused the total loss of

cooperativity for Mg^{2+} and changed the kinetic mechanism to one in which the substrate adds to FBPase before Mg^{2+} and in which all steps equilibrate rapidly relative to the conversion of the ternary complex of enzyme, substrate, and Mg^{2+} to products. It is concluded that Arg 276 is critical for activity and Mg^{2+} cooperativity with FBPase, and it determines the enzyme's kinetic mechanism.

It has been found out that N212, R243, Y244, Y264, and K274 not only are the sites for substrate binding, but also play important role in the binding affinity of inhibitors F26P and AMP.

Kinetic effects of monovalent cations on wild-type and the mutant enzymes were studied. With the wild-type enzyme, K^+ activates the enzyme at low concentration and inhibits the enzyme at high concentrations (2). Li^+ is a linear noncompetitive inhibitor with respect to Mg^{2+} and the substrate. The inhibition effects of monovalent cations, however, are abolished when R276 is mutated to methionine. On the other hand, the activation effect of K^+ is enhanced (600% activation relative to 250% activation with the wild-type enzyme). In the case of E280Q mutant, K^+ inhibits the enzyme activity. The results in this report suggest that R276 is essential for monovalent cation inhibition and E280 is essential for monovalent cation activation. On the basis of this and previous reports, it is postulated that a specific bonding network is responsible for the enzyme to perform

optimal catalysis and communication among metal sites 1 and 3 as well as the allosteric AMP site, which is distal to the metal binding sites. The findings of this report, not only contribute to an understanding of the kinetics and mechanisms of monovalent cation action, but also provide an understanding of signal transmission in FBPase.

Site-directed mutagenesis of amino acid residues at C1C2 (C3C4) interface in the active site domain of porcine liver FBPase were carried out on the basis of crystal structure of the enzyme (3). Mutant FBPases, S123A, D127T, C128G, R243M, and Y258F, were purified to homogeneity and characterized by circular dichroism spectrometry (CD) and initial-rate kinetics. The results showed that the mutations do not significantly affect the k_{cat} of the enzyme and the Hill coefficient for Mg^{2+}. The synergism between AMP and fructose-2,6-bisphosphate (F26P) were not abolished with all the mutant enzymes. The enzyme's affinities for the substrate and regulators, however, were changed in different degrees. Mutation of S123 to alanine reduced K_i for F26P to about 10-fold, K_i for AMP to about 680-fold. The K_a of this mutant enzyme for Mg^{2+}, however, increased to about 3-fold. Replacement of D127 by threonine lowered K_i for AMP to about 327-fold, but K_a for Mg^{2+} increased to about 4-fold. The most interesting mutant is C128G. The mutation caused 48-fold increase in K_m for substrate, 1680-fold increase in K_i for F26P, 34-fold increase in K_i for AMP, and 13-fold increase in

K_a for Mg^{2+}. Similar results were obtained with R243M mutant. The mutation of Y258 to phenylalanine reduced the affinity for F26P to about 47-fold, and that for Mg^{2+} to about 4-fold. This report demonstrates that helix H4 and residues Y258 and R243 from adjacent chain are important in ligand binding. Also, the synergism between AMP and F26P may result from collaborative effort of many amino acid residues.

Future Research

Although the crystal structures of pig kidney FBPase complexed with substrate (4-6), with inhibitors (7-9), and with metal ions (10) have been solved, and site-directed mutagenesis were performed to mutate amino acid residues at the substrate (11), the metal (12,13), and the inhibitor binding sites (14,15) as well as the interfaces between the subunits (16,17), and initial-rate kinetics have been carried out to study the mutant enzymes, the structure-function relationship in FBPase is far from clear. Solutions of the crystal structures of these mutant may help us to understand structure-function relationship in FBPase at the molecular level. In addition, solutions of crystal structures of this enzyme complexed with its products may help us to understand the chemical mechanisms in both forward and reverse directions.

References

1. Zhang, Y., Liang, J-Y, Huang, S., Ke, H., and Lipscomb, W.N. (1993) Biochemistry **32**, 1844-1857.
2. Zhang, R., Villeret, V., Lipscomb, W.N., and Fromm, H.J. (1996) Biochemistry **35**, 3038-3043.
3. Xue, Y., Huang, S., Liang, J-Y, Zhang, Y., and Lipscomb, W.N. (1994) Proc. Natl. Acad. Sci. USA **91**, 12482-12486.
4. Ke, H., Liang, J-Y, Zhang, Y., and Lipscomb, W.N. (1991) Biochemistry **30**, 4412-4420.
5. Ke, H., Thorpe, C.M., Seaton, B., Marcus, F., and Lipscomb, W.N. (1989) Proc. Natl. Acad. Sci. USA **86**, 1475-1479.
6. Villeret, V., Huang, S., Fromm, H.J., and Lipscomb, W.N. (1995) Proc. Natl. Acad. Sci. USA **92**, 8916-8920.
7. Shyur, L., Zhang, R., Fromm, H.J. (1995) Arch. Biochem. Biophys. **319**, 123-127.
8. Zhang, R., Honzatko, R.B., Fromm, H.J. (1997) Biochemistry, submitted.
9. Chen, L., Hegde, R., Chen, M., and Fromm, H.J. (1993) Arch. Biochem. Biophys. **307**, 350-354.
10. Gidh-Hain, M., Zhang, Y., Liang, J-Y, Huang, S., Kim, J., Pilkis, S.J., El-Maghrabi, M., and Lipscomb, W.N. (1994) J. Biol. Chem. **269**, 27732-27738.
11. Chen, M., Chen, L., and Fromm, H.J. (1994) J. Biol. Chem. **269**, 5554-5558.
12. Zhang, R., Shyur, L., and Fromm, H.J. (1997) Arch. Biochem. Biophys., submitted.

13. Shyur, L., Aleshin, A.E., and Fromm, H.J. (1996) Biochemistry 35, 7492-7498.

ACKNOWLEDGEMENTS

I would like to thank Dr. Herbert J. Fromm, my major professor, for his guidance, help, and support during the course of my Ph.D program. I am very grateful to my POS committee members, Dr. Richard Honzatko, Dr. James Thomas, Dr. Basil Nicholau, Dr. Eric Henderson, Dr. Ted Huiatt, and Dr. Kristen Johansen for their guidance, advice, and time.

I thank my parents, who raised and nurtured me; my wife and my daughter, for their patience and sacrifice.

Many thanks also go to my colleagues, for their help and encouragement, particularly in the beginning of my graduate research.

VITA

NAME OF AUTHOR: Rulin Zhang

DATE AND PLACE OF BIRTH: January 1, 1961, Henan, PR China

DEGREE AWARDED:
B.S. in chemistry, Zhengzhou University (PR China), 1983
M.S. in biology, Truman State University (USA), 1993
Ph.D. in biochemistry, Iowa State University (USA), 1997

HONORS AND AWARDS:
Teaching Excellent Award, Iowa State University, 1996
Premium for Academic Excellence Award, Iowa State University, 1993
Sigma XI Grant in Aid, Truman State University, 1993
Capillary Electrophoresis Symposium Traveling Award, 1993
Excellent Student Awards, Zhengzhou University, 1979-1983

PROFESSIONAL EXPERIENCES:
Research Assistant, Henan Academy of Agricultural Sciences, PR China, 1983-1989
Visiting Scholar, Meggler Milk Industry, Germany, 1989-1991
Visiting Scholar, Truman State University, 1991
Teaching and Research Assistant, Truman State University, 1992-1993
Research Assistant, Iowa State University, 1993-1997

SELECTED PROFESSIONAL PUBLICATIONS

1. Zhang, R., Villeret, V., Lipscomb, W.N., and Fromm, H.J. (1996) Biochemistry **35**, 3038-3043.
2. Zhang, R., and Fromm, H.J. (1995) Biochemistry **34**, 8190-8195.
3. Zhang, R., Chen, L., Villeret, V., and Fromm, H.J. (1995) J. Biol. Chem. **270**, 54-58.
4. Shyur, L., Zhang, R., and Fromm, H.J. (1995) Arch. Biochem. Biophys. **319**, 123-127.
5. Zhang, R., Shi, H., and Ma, Y. (1994) J. Microcol. Sep. **6**, 217-221.
6. Shi, H., Zhang, R., Chandrasekher, G., and Ma Y. (1994) Journal of Chromatography **680**, 653-658.
7. Zhang, R., Cooper, C., and Ma, Y. (1993) Analytical Chemistry **65**, 704-706.
8. Ma, Y., and Zhang, R. (1992) Journal of Chromatography **625**, 341-348.
9. Wen, G., Wang, W., and Zhang, R. (1990) J. of Catalysis **2**, 101-105.
10. Wen, G., Wang, W., and Zhang, R. (1990) Chemical Researches **1**, 1-7.
11. Zhang, R. (1988) Henan Agri. Sci. **10**, 12-14.
12. Zhang, R. (1987) Henan Agri. Sci., **1**, 1-7.